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Is serotonin uptake by peripheral tissues sensitive to hypoxia exposure?

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Abstract In the Gulf toadfish (*Opsanus beta*), the serotonin (5-HT) transporter (SERT) is highly expressed in the heart, and the heart and gill both demonstrate the capacity for SERT-mediated uptake of 5-HT from the circulation. Because 5-HT is a potent vasoconstrictor in fish, we hypothesized that hypoxia exposure may increase 5-HT uptake by these tissues—and increase excretion of 5-HT—to prevent branchial vasoconstriction that would hamper gas exchange. Spot sampling of blood, bile, and urine revealed that fish exposed to chronic hypoxia (1.83 ± 0.12 mg·L^{-1} O_2 for 24–26 h) had 41% lower plasma 5-HT in the ventral aorta (immediately following the heart) than in the hepatic vein (immediately before the heart), suggesting enhanced cardiac 5-HT uptake during hypoxia. 5-HT concentrations in the bile were greater than those in the urine, but there were no effects of acute (1.31 ± 0.06 mg·L^{-1} O_2 for 25 min) or chronic hypoxia on 5-HT levels in these fluids. In 5-HT radiotracer experiments, the presence of tracer in the bile decreased upon hypoxia exposure, but, surprisingly, neither acute nor chronic hypoxia-induced changes in [^{3}H]5-HT uptake in the heart, gill, or other tissues. Given the likely impact of the hypoxia exposure on metabolic rate, future studies should examine the effects of a milder hypoxia exposure on 5-HT uptake into these tissues and the role of 5-HT degradation.

Keywords 5-HT · 5-Hydroxytryptamine · SERT · Heart · Gill · *Opsanus beta*

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Introduction

Serotonin (5-HT; 5-hydroxytryptamine) is a monoamine neurochemical that has been documented to play a role in various physiological processes in fish including reproduction (reviewed by Prasad et al. 2015), the endocrine stress response (Höglund et al. 2002; Lim et al. 2013; Medeiros et al. 2014), sex differentiation (reviewed by Senthilkumaran et al. 2015), muscle contraction (Slánský et al. 1996; Velarde et al. 2010), and appetite (De Pedro et al. 1998; Ortega et al. 2013; Perez Maceira et al. 2014). The gill of the Gulf toadfish (*Opsanus beta*) expresses the serotonin transporter (SERT) at levels similar to those in the brain and removes 5-HT from the circulation in a SERT-dependent manner (Amador and McDonald 2018a, b), supporting earlier work by Olson (1998) that showed a capacity for 5-HT uptake in the gill of rainbow trout. Perhaps even more intriguing, the toadfish heart contains up to 30 times more SERT transcript than any other tissue (Amador and McDonald

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2018a), consistent with the apparently high SERT mRNA expression in the heart of the goldfish (Mennigen et al. 2010). The toadfish atrium and ventricle take up 5-HT from the circulation rapidly and to a greater extent than any other tissue (Amador and McDonald 2018b). While 5-HT uptake by the toadfish gill and heart is largely SERT-mediated, promiscuous transporters, such as the norepinephrine and dopamine transporters, and low-affinity transporters, such as the organic cation transporter and the plasma membrane monoamine transporter, play roles in 5-HT uptake under normal conditions and become increasingly important upon SERT inhibition (Amador and McDonald 2018b).

The adaptive significance of branchial and cardiac SERT-mediated 5-HT uptake from the circulation has not been investigated. However, when present in the branchial vasculature, 5-HT is a potent vasoconstrictor (Fritsche et al. 1992; Janvier et al. 1996; McDonald et al. 2010; Sundin et al. 1998, 1995; Sundin and Nilsson 2000; reviewed by Pelster and Schwerte 2012). Furthermore, Fritsche et al. (1992) and Sundin et al. (1995) demonstrated that 5-HT within the branchial circulation results in vasoconstriction that reduces gas exchange—an action that could be particularly detrimental during hypoxia exposure. We suggest that the gill—and particularly the heart, which immediately precedes the gill within the teleost circulatory loop (Laurent 1984)—may be well situated to control circulating levels of 5-HT via SERT or other transporters, thereby preventing unchecked vasoconstriction that could reduce O_2 uptake. The 5-HT uptake capacity of the gill and heart could be particularly important during hypoxia, when gas exchange across the gill must be optimal to ensure survival. This hypothesis surmises that circulating 5-HT exerts a tonic vasoconstriction on branchial and systemic blood vessels that can be alleviated upon removal from the circulation. While this has not been shown directly, systemic inhibition of SERT (i.e., preventing the removal of 5-HT from the circulation) does result in an increase in caudal arterial blood pressure (P_{CA}) (Panlilio et al. 2016), which could suggest a combined branchial and systemic vasoconstriction. Furthermore, when pre-treated with ketanserin (a 5-HT_2 receptor antagonist), toadfish isolated perfused gills have a ventral aortic inflow pressure and vascular resistance that is 40–60% that of controls (McDonald et al. 2012), suggesting branchial vasodilation when the action of 5-HT is prevented. Notably, the cardiovascular response to the systemic inhibition of SERT—an increase in P_{CA} and tachycardia—opposes the cardiovascular response to hypoxia (Panlilio et al. 2016), and, not surprisingly, inhibiting SERT attenuates the hypoxia response in toadfish (Panlilio et al. 2016), suggesting the importance of 5-HT uptake during hypoxia. Whether 5-HT uptake by the teleost gill or heart changes during hypoxia has never been investigated.

5-HT uptake, metabolism, and excretion by other tissues, such as the intestine, liver, and kidney (Caamaño-Tubío et al. 2007; Edwards et al. 1986), could provide another important mechanism for 5-HT inactivation during hypoxia in concert with cardiac and/or branchial uptake. Indeed, SERT mRNA expression and 5-HT uptake have been measured in all toadfish tissues examined (Amador and McDonald 2018a, b), suggesting the potential for widespread 5-HT control. Furthermore, inhibition of SERT reduces overall excretion of 5-HT and/or its metabolites in toadfish, indicating the importance of SERT in 5-HT excretion (Amador and McDonald 2018b). Thus, further exploration of the effects of hypoxia on SERT-mediated 5-HT uptake, metabolism, and excretion is warranted.

Understanding the potential roles of SERT-mediated uptake and excretion in the hypoxia response is important not only to increase the body of knowledge regarding hypoxia tolerance but also because there is a growing presence of fluoxetine (FLX) and other selective serotonin reuptake inhibitor antidepressants (SSRIs), which target SERT, as pollutants in the aquatic environment (Kolpin et al. 2002; Brooks et al. 2005; Focazio et al. 2008; Vasskog et al. 2008). Thus, the objective of the current study was to elucidate whether 5-HT uptake by the gill, heart, and excretory tissues is enhanced during exposure to hypoxia in the Gulf toadfish. We hypothesized that (1) plasma 5-HT would decrease after the passage of the blood through the heart and again after passage through the gill, reflecting 5-HT uptake by these tissues; (2) hypoxia would increase 5-HT uptake from the circulation and excretion of 5-HT and its metabolites; and (3) inhibition of SERT and other transporters would decrease the hypoxia-induced increases in 5-HT uptake and excretion.

Materials and methods

Experimental animals

Gulf toadfish (*O. beta*, Goode & Beane 1880) were obtained from local shrimpers who caught them as bycatch in Biscayne Bay, Florida, during the fall and winter of 2017/2018 (Florida Fish and Wildlife Conservation Commission Special Activity License #SAL-16–0729-SR). The fish were exposed to freshwater for 15 min upon their arrival at the laboratory, returned to seawater, and treated with final concentrations of 0.1 $mg{\cdot}L^{-1}$ malachite green in 30 $mg{\cdot}L^{-1}$ formalin (Rid Ich Plus, Kordon, Hayward, CA, USA) on that day and on the two subsequent days to treat and prevent ectoparasite infection. The fish were housed in aerated, flow-through 20-gal aquaria that received filtered seawater from Biscayne Bay. The water temperatures ranged from 20 to 23 °C, and the fish were fed raw shrimp weekly to satiation. All protocols were conducted under the approval of the University of Miami Institutional Animal Care and Use Committee and conformed to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The sexes were recorded for approximately half of the experimental animals (distributed among groups); among the 36 fish for which sex was recorded, males overwhelmingly predominated (32/36; 89%). Given the specific identification and sequestration of females by another researcher in the laboratory during the same time period, it is likely that the remaining experimental animals for which sex was not recorded were also overwhelmingly male. The few confirmed females in this experiment were all in separate groups, and no outliers were identified in any of the corresponding groups of data (ROUT method, $Q = 1\%$).

Experimental series

Series i: 5-HT spot sampling

Fish fed 1 day previously were placed into individual aerated boxes with flow-through seawater. At $t = 0$, two groups of fish, a normoxia group ($n = 6$) and an acute hypoxia group ($n = 6$), were held in water bubbled vigorously with air (20.9% O_2); a third group of fish, a chronic hypoxia group ($n = 6$), was held in water bubbled vigorously with a 2.5% O_2 balance N_2 gas mixture purchased pre-made from Airgas (Radnor, PA). The initial dissolved O_2 in all the boxes was measured with a ProODO Optical Dissolved O_2 instrument (YSI Incorporated, Yellow Springs, OH, USA) (the concentrations were 7.26 ± 0.03 $mg{\cdot}L^{-1}$, 7.16 ± 0.04 $mg{\cdot}L^{-1}$, and 2.76 ± 0.35 $mg{\cdot}L^{-1}$ for the normoxia, acute hypoxia, and chronic hypoxia groups, respectively). Water flow was then switched off. Approximately 24–26 h after being placed in the boxes, the fish in the acute hypoxia group were exposed to hypoxia for 25 min via vigorous bubbling with the 2.5% O_2 balance N_2 mixture. Thus, fish in the normoxia group were exposed to normoxia (20.9% O_2) for 24–26 h, fish in the acute hypoxia group were exposed to normoxia for 24–26 h followed by hypoxia (2.5% O_2 balance N_2) for 25 min, and fish in the chronic hypoxia group were exposed to hypoxia for 24–26 h. The final dissolved O_2 levels for the normoxia, acute hypoxia, and chronic hypoxia groups were 7.42 ± 0.09 $mg{\cdot}L^{-1}$, 1.31 ± 0.06 $mg{\cdot}L^{-1}$, and 1.83 ± 0.12 $mg{\cdot}L^{-1}$, respectively. After all exposures were complete, a blood sample was obtained from each fish through caudal puncture using a syringe fitted with a 23-G needle and rinsed with heparinized saline. The fish were then anesthetized in 1 $g{\cdot}L^{-1}$ tricaine methanesulfonate (MS-222; Western Chemical, Ferndale, WA, USA) (except for one fish for which the anesthetic was not aerated, the normoxia group fish were anesthetized in MS-222 bubbled with air). The gall bladder and urinary bladder were harvested, and blood samples were taken from the hepatic vein(s) and the bulbus arteriosus/ventral aorta. All samples were immediately placed on ice until harvesting was complete. The gallbladders and urinary bladders were then punctured, and all samples were centrifuged for 10 min at 16,100 g and 15 °C. The plasma, bile, and urine supernatants were collected into new tubes, flash-frozen, and stored at − 80 °C for later analysis of 5-HT concentrations.

Series ii: 5-HT uptake experiment

Fish fed 5–9 days previously ($n = 54$) were anesthetized in 1 $g{\cdot}L^{-1}$ MS-222 and implanted with caudal vessel catheters (Intramedic PE 50 tubing; Becton Dickinson, Franklin Lakes, NJ, USA) filled with heparinized saline (150 mM NaCl with 50 $IU{\cdot}ml^{-1}$ sodium heparin; Sigma-Aldrich, St. Louis, MO,

USA) as described previously (Wood et al. 1997). Intraperitoneal (IP) catheters (Intramedic PE 160 tubing; Becton Dickinson) were implanted as described by McDonald and Walsh (2004) and filled with peanut oil. Each IP catheter was further secured with a suture placed directly anterior to the sutured caudal vessel catheter incision, and the catheter was sealed with putty. After surgery, the fish were placed in individual aerated boxes with flow-through seawater and allowed to recover undisturbed overnight.

After recovery, water flow was switched off, and the boxes for a subset of fish (chronic hypoxia group; $n=18$) were aerated with a 2.5% O_2 balance N_2 mixture to establish chronic (24 h) hypoxia. The boxes for the two other groups (normoxia and acute hypoxia groups; $n=18$ per group) remained aerated with air. Meanwhile, coconut oil and drug mixtures were prepared as described previously (Amador and McDonald 2018b). Briefly, bupropion hydrochloride (BUP; VWR International, Radnor, PA, USA), a norepinephrine and dopamine transporter inhibitor; decynium-22 (D-22; VWR International), an organic cation transporter and plasma membrane monoamine transporter inhibitor; and FLX (as FLX hydrochloride; Toronto Research Chemicals, North York, ON, CAN), a selective serotonin reuptake inhibitor, dissolved in 100% ethanol were overlaid on a coconut oil vehicle (5 µl·g fish^{-1}) for final doses of 10 µg·g fish^{-1} BUP, 0.01 µg·g fish^{-1} D-22, and 50 µg·g fish^{-1} FLX (+B+D+FLX treatment), respectively. A separate tube contained BUP and D-22 in the same doses overlaid on the coconut oil vehicle (+B+D treatment). The control treatment consisted of coconut oil only. A FLX alone treatment was not used, as a previous study demonstrated that FLX alone tends to result in an increase in 5-HT uptake, due to the compensation by the other nonselective transporters that are blocked by BUP and D-22 (Amador and McDonald 2018b). The dosage of FLX was selected based on our previous studies with FLX alone (Morando et al. 2009; Panlilio et al. 2016; Amador and McDonald 2018b). This same dose combination of FLX, BUP, and D-22 was used in a previous study on toadfish (Amador and McDonald 2018b) and inhibited 5-HT uptake. At the time of that study, effective concentrations for BUP and D-22 in fish had not been established, but BUP at this dose was found to elicit antidepressant effects in rats (Cooper et al. 1980), and D-22 at this dose was the lowest effective therapeutic dose in rats (Marcinkiewcz and Devine 2015; Baganz et al. 2008). Ethanol was added to the control and +B+D tubes to ensure an equivalent overlay volume for all treatments. The tubes were left open overnight at 30 °C to evaporate the ethanol and retain the vehicle in a liquid state. After evaporation, the tubes were vortexed, and a syringe fitted with an 18-G needle was used to mix the tube contents into a slurry.

Bubbling with the 2.5% O_2 balance N_2 mixture was initiated for the fish in the acute hypoxia group ($t=-15$ min, 15-min before drug treatment at t=0) 24 h after the water flow was switched off. After this time, all fish were injected via IP catheter with coconut oil only, +B+D, or +B+D+FLX ($t=0$; $n=6$ per treatment per oxygen scheme). The IP catheters were then flushed with 250–400 µl of peanut oil to ensure full drug delivery into the fish. At $t=5$ min, the fish were injected with 0.05 µCi·g fish^{-1} [^{3}H]5-HT creatinine sulfate (36.5 Ci·mmol^{-1}; American Radiolabeled Chemicals, St. Louis, MO, USA) via caudal vessel catheter using a gastight Hamilton syringe (Hamilton Company, Reno, NV, USA) fitted with a 23-G needle. A ~ 150-µl pre-sample of blood was collected before isotope injection and reinjected after isotope injection to ensure full delivery of the isotope. At $t=7$ min (after approximately 22 min of hypoxia exposure for the acute hypoxia group), a ~ 150-µl blood sample was collected from each fish and placed on ice. The remaining blood was centrifuged for 10 min at 16,100 × g at 4 °C, and 20-µl aliquots of plasma were added to scintillation vials containing 4 ml of UltimaGold liquid scintillation cocktail (PerkinElmer, Waltham, MA, USA). Additional aliquots of 60–70 µl of plasma were flash-frozen in liquid nitrogen and stored at −80 °C for later analysis of plasma 5-HT concentrations.

After blood sample collection, the fish were quickly anesthetized in 3 g·L^{-1} MS-222. The final dissolved O_2 levels for the normoxia, acute hypoxia, and chronic hypoxia groups were 5.44 ± 0.23 mg·L^{-1}, 1.32 ± 0.16 mg·L^{-1}, and 1.08 ± 0.06 mg·L^{-1}, respectively. After anesthesia, the heart was quickly removed (to stop the circulation of isotope), flushed with 150 mM NaCl, and gently pressed to remove residual blood from the lumen. The atrium (including the sinus venosus), ventricle, and bulbus arteriosus were separated and collected. The brain, gill arches, kidneys, liver, bile, and urine were also collected. The tissues were weighed, and 1 N nitric acid was added

to each tissue in a 5:1 ratio (5 µl acid per 1 mg tissue). The tissues were vortexed and then digested for 48 h in a 70 °C water bath (with an additional vortex after the first 24 h of digestion). After digestion, the tissues were again vortexed and were centrifuged for 5 min at 1500 g; 20 µl of supernatant was collected from each sample and added to a scintillation vial containing 4 ml of UltimaGold.

Analytical techniques and calculations

The scintillation vials containing plasma and digested tissue supernatants were shaken vigorously, and radioactivity was counted in a Tri-Carb 2910TR liquid scintillation counter (PerkinElmer) with QuantaSmart software (PerkinElmer). 5-HT concentrations in plasma and in bile and urine (*Series i* only) were measured using an ELISA kit (ALPCO Diagnostics, Salem, NH, USA) as described previously for toadfish (Morando et al. 2009). The intra-assay coefficient of variability (CV) was 2.9%, and the inter-assay CV was 3.7%.

In *Series i*, the clearance ratios for bile and urine were calculated based on the equation

$$R = \frac{m}{b} \quad (1)$$

where R is the clearance ratio, m is the measured 5-HT concentration (in ng·ml^{-1}) in either the bile or the urine, and b is the 5-HT concentration measured in the plasma of the caudal vessel blood sample (in ng·ml^{-1}).

For *Series ii* experiments, the plasma-specific activity was calculated based on the following equation:

$$SA_P = \frac{a}{b} \quad (2)$$

where SA_P is the plasma-specific activity, a is the plasma radioactivity (in dpm·ml^{-1}), and b is the total 5-HT concentration in the plasma (in ng·ml^{-1}). 5-HT uptake was then calculated for each tissue as follows:

$$U_{sample} = \frac{c}{SA_P} \quad (3)$$

where U_{sample} is the tissue 5-HT uptake and c is the radioactivity (dpm) per gram tissue (wet weight) (Amador and McDonald 2018b).

Statistics

The data were tested for normality with the Shapiro–Wilk test; non-normal data were multiplied by 1000 (if necessary, to ensure values > 1) and log-transformed to normality, if possible. For *Series i*, the data were analyzed by two-way ANOVA with Tukey's multiple comparisons test or, for pooled data, with two-tailed unpaired student's t tests. For *Series ii*, data that met the normality assumptions were analyzed using two-way ANOVA with Tukey's multiple comparisons test. For data that could not be transformed to normality for two-way ANOVA, pooled data were analyzed for the effect of drug treatment or oxygen scheme by one-way ANOVA. The effects of oxygen scheme within each drug treatment group (for data that could not be analyzed by two-way ANOVA) were analyzed by one-way ANOVA with Tukey's multiple comparisons test or by Kruskal–Wallis test with Dunn's multiple comparisons test. The data are presented as the means ± SEM, and a p value < 0.05 was defined as the threshold of statistical significance.

Results

There were no significant overall differences in measured 5-HT concentrations in plasma samples taken from the caudal vessel, hepatic vein, and bulbus arteriosus/ventral aorta, nor was there an overall effect of acute hypoxia on the 5-HT concentrations of the blood plasma from these different locations (Fig. 1). However, when fish were exposed to chronic hypoxia, plasma 5-HT in the ventral aorta (which leads away from the heart to the gill) was 41% lower than that in the hepatic vein (which leads to the heart from the liver) (Fig. 1). Neither acute nor chronic hypoxia affected 5-HT concentrations in the bile and urine, but 5-HT concentrations were 33% lower in the urine than in the bile of toadfish when oxygen schemes were pooled, and the concentrations of 5-HT in the bile and urine were 5.0–6.2-fold and 3.3–4.1-fold higher than those in the plasma, respectively (Fig. 1). The bile and urine clearance ratios for 5-HT were > 1 for the toadfish in all oxygen schemes (Table 1). The urine and bile clearance ratios for 5-HT did not change with oxygen regime and were not significantly different from each other except in fish exposed to acute hypoxia, in which the urine clearance ratio for

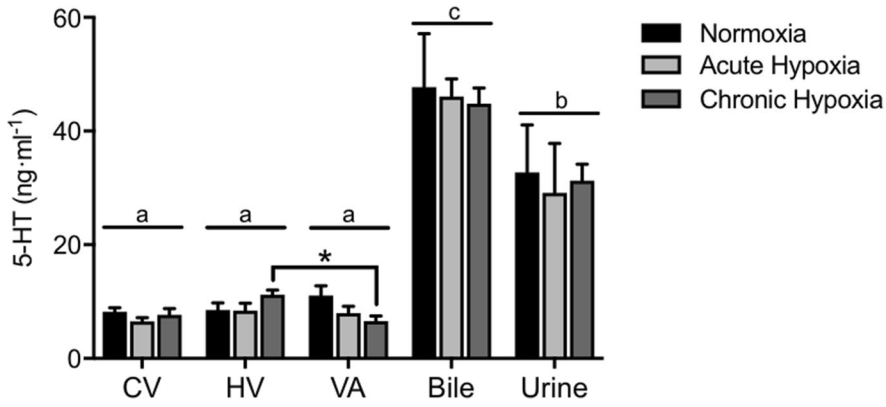

Fig. 1 Measured 5-HT concentrations in plasma sampled from the caudal vessel (CV), hepatic vein (HV), and ventral aorta (VA) and in the bile and urine of toadfish after exposure to normoxia, acute hypoxia, or chronic hypoxia. The data are the mean ± 1 SEM. Bars not sharing a lowercase letter are significantly different from each other; an asterisk (*) denotes a significant difference between two bars ($p < 0.05$; two-way ANOVA with Tukey's multiple comparisons test and student's *t* test; $n = 5$–6 per group)

Table 1 Bile and urine 5-HT clearance ratios for fish held in normoxia or exposed to acute or chronic hypoxia

Oxygen scheme	5-HT clearance ratio	
	Bile	Urine
Normoxia	6.08 ± 1.52 (6)	4.92 ± 1.74 (4)
Acute hypoxia	7.43 ± 0.98 (6)	4.28 ± 1.01 (6)*
Chronic hypoxia	6.42 ± 1.45 (4)	4.30 ± 0.82 (5)

The data are the mean ± 1 SEM (n). An asterisk (*) indicates a significant difference between fluids ($p < 0.05$, student's *t* test)

5-HT was 32% lower than the bile clearance ratio (Table 1).

There was no effect of oxygen scheme on measured plasma 5-HT concentrations under control/no-drug conditions or when fish were treated with either + B + D or + B + D + FLX (Fig. 2). However, there was a significant overall effect of drug treatment; measured plasma 5-HT in + B + D + FLX fish, regardless of oxygen regime, was 1.6 and 1.4-fold

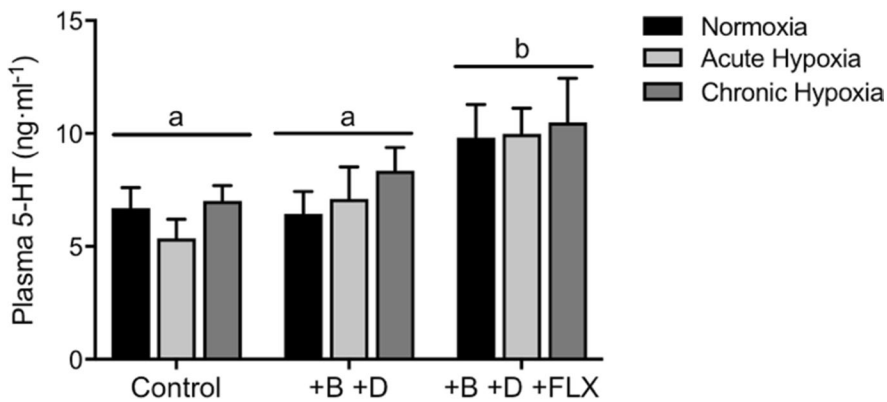

Fig. 2 Plasma 5-HT concentration after control or drug treatment and exposure to normoxia, acute hypoxia, or chronic hypoxia. The data are the mean ± 1 SEM. Sets of bars that do not share a lowercase letter are significantly different from each other ($p < 0.05$; two-way ANOVA with Tukey's multiple comparisons test; $n = 6$ per group except for + B + D chronic hypoxia group, $n = 5$)

greater than that in control and + B + D fish, respectively (Fig. 2).

5-HT uptake in the atrium (which includes the sinus venosus) and the ventricle was nearly an order of magnitude higher than that in the bulbus arteriosus, and the uptake in the atrium appeared higher than that in the ventricle (Fig. 3A *cf.* 3B *cf.* 3C) but was not significantly different. As in the blood, neither acute nor chronic hypoxia affected 5-HT uptake in the heart under control/no-drug conditions or when the fish

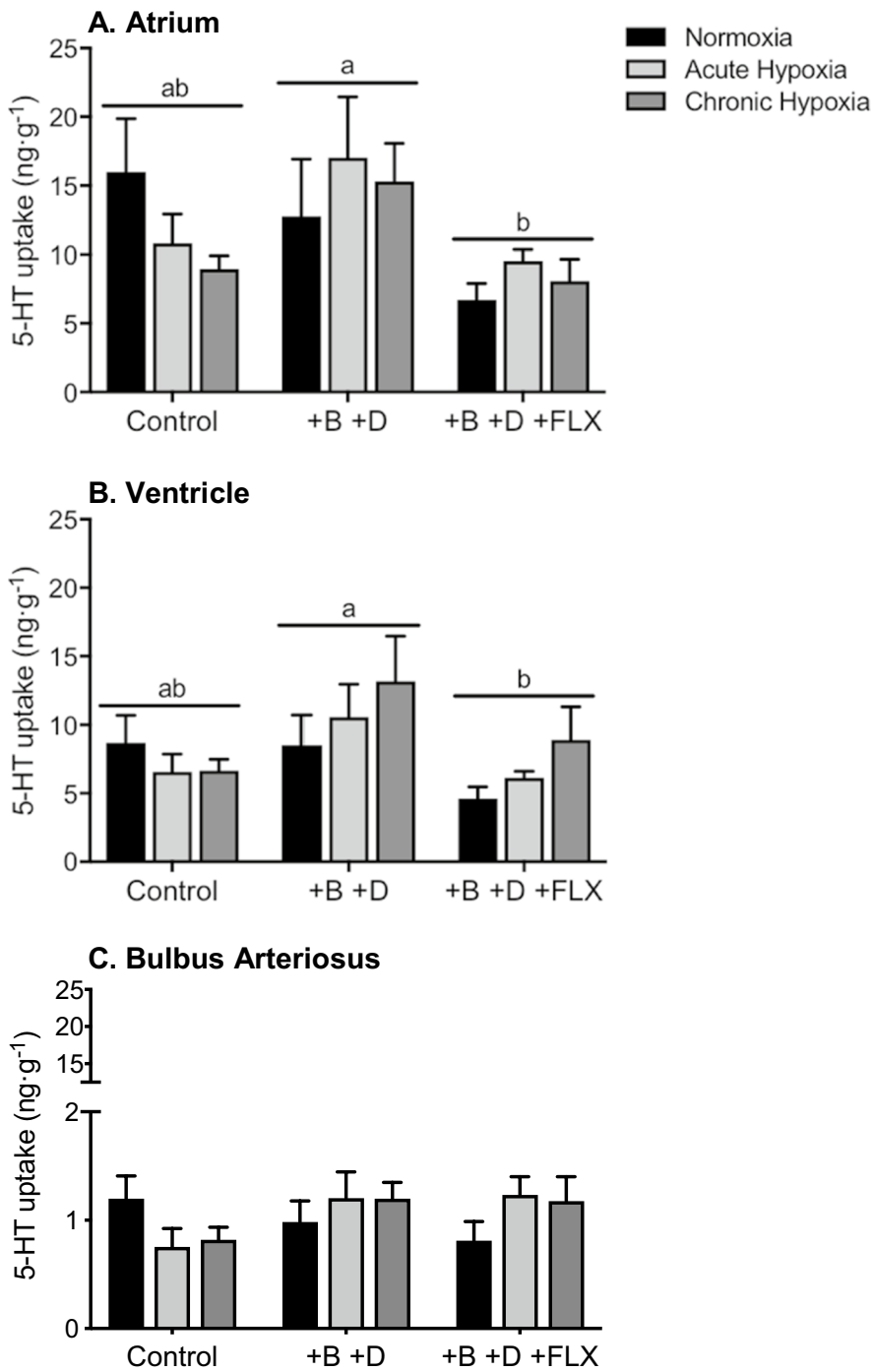

Fig. 3 5-HT uptake in the **A** atrium, **B** ventricle, and **C** bulbus arteriosus in toadfish after control or drug treatment and exposure to normoxia, acute hypoxia, or chronic hypoxia. The data are the mean ± 1 SEM. Sets of bars that do not share a lowercase letter are significantly different from each other ($p < 0.05$; two-way ANOVA with Tukey's multiple comparisons test; $n = 6$ per group except for + B + D chronic hypoxia group, $n = 5$

were treated with either + B + D or + B + D + FLX (Fig. 3A–C). Compared to treatment with + B + D alone, treatment with + B + D + FLX caused a 46% reduction in 5-HT uptake in the atrium and a 38% reduction in the ventricle, regardless of oxygen regime (Fig. 3A, B). There was no effect of drug treatment on the bulbus arteriosus (Fig. 3C). There was no effect of hypoxia or drug treatment on 5-HT uptake in the brain, and overall uptake of 5-HT by the brain was the lowest for any tissue examined (Fig. 4A). 5-HT uptake in the gill was also unaffected by hypoxia, but 5-HT uptake in the gill in + B + D fish was 1.5-fold greater than that in control fish, and uptake in + B + D + FLX fish was 44% lower than that in fish treated with + B + D alone (Fig. 4B).

In the kidneys and liver, there were no significant effects of hypoxia or drug treatment when drug treatment groups and oxygen schemes were pooled, respectively (Fig. 5A, B). There were also no significant effects of hypoxia within drug treatment groups (Fig. 5A, B). In the bile, there was not a significant effect of drug treatment compared to controls when oxygen schemes were pooled, and there was no effect of oxygen regime on either drug treatment group (Fig. 5C). However, in control fish, chronic hypoxia caused a 47% decrease in biliary [^{3}H] accumulation compared to normoxia (Fig. 5C).

Discussion

In contrast to the hypothesis that 5-HT uptake by the heart and gill would decrease circulating 5-HT in certain regions of the circulatory system, there were no measurable differences in plasma 5-HT among the spot sampling locations in normoxic fish. However, fish that were exposed to chronic hypoxia had significantly lower plasma 5-HT in the ventral aorta (which leads from the heart to the gill) than that in the hepatic vein (which leads from the liver to the heart), suggesting uptake by the heart during the extended hypoxia challenge. This finding is consistent with the

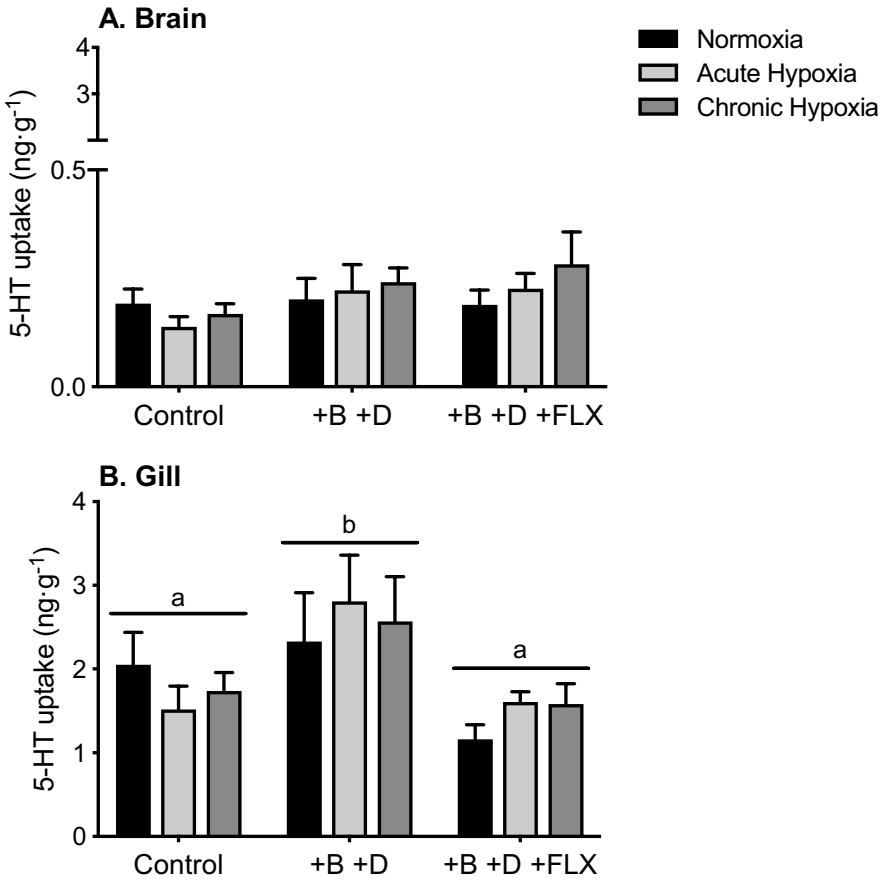

Fig. 4 5-HT uptake in the **A** brain and **B** gill in toadfish after control or drug treatment and exposure to normoxia, acute hypoxia, or chronic hypoxia. The data are the mean ± 1 SEM. Sets of bars that do not share a lowercase letter are significantly different from each other ($p < 0.05$; two-way ANOVA with Tukey's multiple comparisons test; $n = 6$ per group except for + B + D chronic hypoxia group, $n = 5$

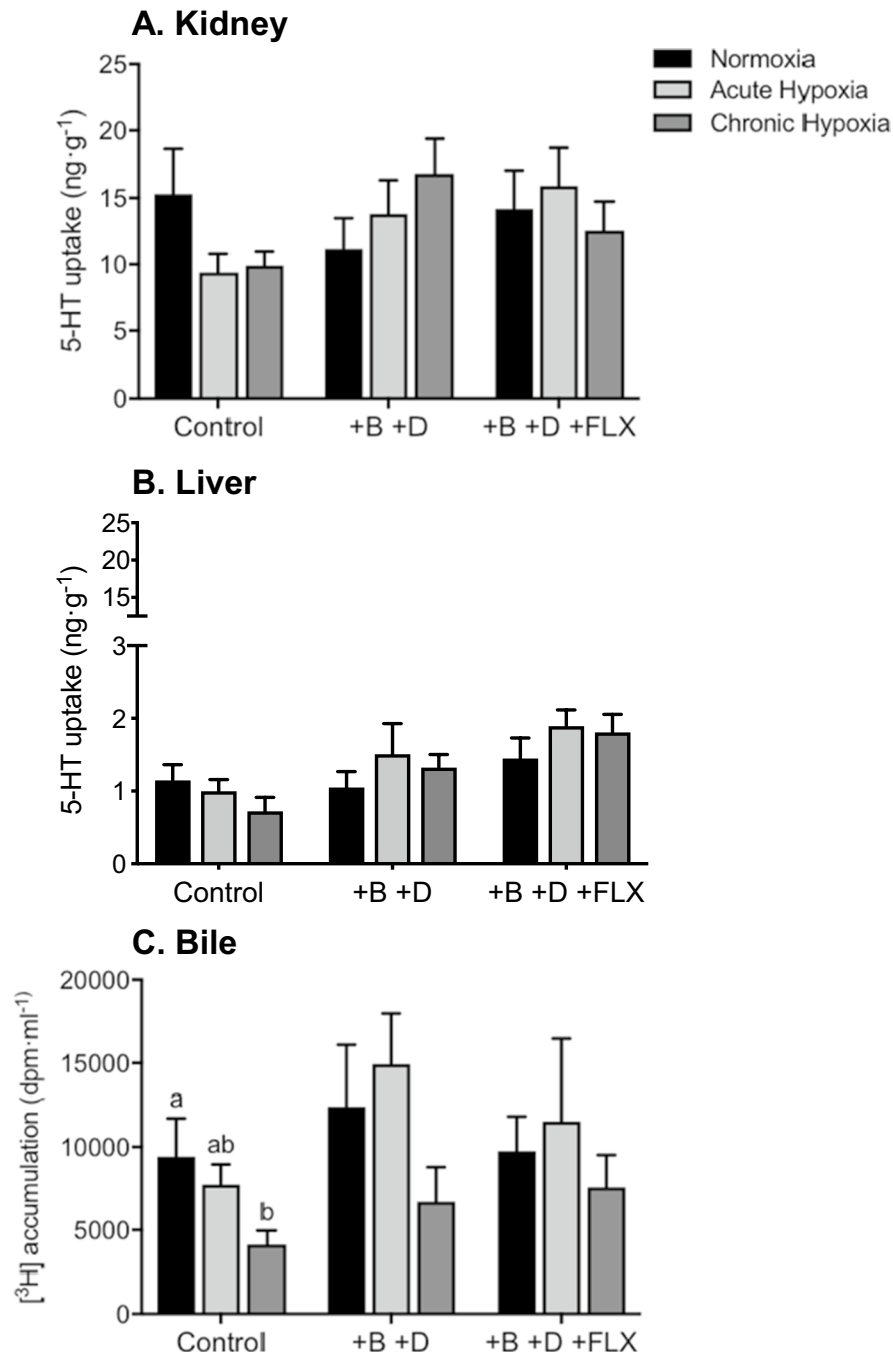

Fig. 5 5-HT uptake in the **A** kidney and **B** liver and **C** [^{3}H] accumulation in the bile of toadfish after control or drug treatment and exposure to normoxia, acute hypoxia, or chronic hypoxia. The data are the mean ± 1 SEM. Bars that do not share a lowercase letter are significantly different from each other ($p < 0.05$; one-way ANOVA with Tukey's multiple comparisons test; $n = 5$–6 per group)

elevated SERT mRNA expression and 5-HT uptake in the heart (Amador et al. 2018; Amador and McDonald 2018a, b). Furthermore, the magnitude by which passage through the heart reduced plasma 5-HT concentrations in the fish exposed to chronic hypoxia was approximately 4.6 ng·ml^{-1}. This change is well within the range that could cause branchial vasoconstriction; Sundin et al. (1998) reported branchial vasoconstriction in another teleost, the borch, after administration of only 0.1 nmol·kg^{-1} 5-HT, which would produce an estimated increase in plasma 5-HT of 0.4 ng·ml^{-1} in the fish of the current study, given their average masses and previous estimates of fish plasma volume (Smith 1966). Thus, the spot sampling result supports

our hypothesis that cardiac 5-HT uptake should be particularly important during hypoxia to prevent impaired gas exchange at the gill that could occur as a result of 5-HT-mediated branchial vasoconstriction (Fritsche et al. 1992). That no reductions were measured between the ventral aorta (located before the gill) and the caudal vessel (located after the gill) even after chronic hypoxia could suggest that the heart is relatively more important for 5-HT regulation during hypoxia than the gill; however, changes in plasma 5-HT due to gill extraction may have been present but rendered insignificant by other processes (e.g., release of 5-HT synthesized by other tissues or dilution due to changes in blood volume or blood components) by the time the blood reached the caudal vessel. Blood sampling from the dorsal aorta (immediately following the gill in the circulatory loop) would minimize this possibility; however, this vessel is fragile and difficult to access in the toadfish.

The substantially greater 5-HT content in the bile and urine spot samples than in the plasma, measured using ELISA, should be validated using another approach, for example, high-pressure liquid chromatography (HPLC; Khan and Thomas 1996; Kupiec 2004; Cartolano et al. 2017). With that limitation in mind, the high biliary and urinary 5-HT content as well as the clearance ratios > 1 for both bile and urine suggests that the biliary and renal pathways both have the ability to concentrate 5-HT. The presence of 5-HT in these fluids is consistent with the accumulation of [^{3}H] in toadfish bile and urine reported by Amador and McDonald (2018b) after [^{3}H]5-HT injection and suggests that a portion of 5-HT is excreted unchanged in toadfish, as it is in mammals (McIsaac and Page 1959). The higher 5-HT content in bile may suggest that the biliary route plays a significant role in 5-HT excretion. Alternatively, it is possible that the 5-HT in the bile is not destined for excretion at all but rather is being delivered to the intestine after synthesis in (or uptake by) the liver. The rate-limiting enzyme in 5-HT synthesis, tryptophan hydroxylase, has been measured in the livers of several teleosts, while the intestines of several teleosts, unlike mammalian intestines (Anderson and Campbell 1988; Kiliaan et al. 1989; Olsson et al. 2008; reviewed in Mawe and Hoffman 2013), lack this enzyme (Nagai et al. 1997)—although the intestine of one species, the sand flathead, has been found to synthesize 5-HT from precursors (Anderson et al. 1989). Nevertheless, the levels of unmetabolized 5-HT in the bile and urine were not affected by hypoxia exposure, suggesting that neither excretion of unchanged 5-HT nor potential 5-HT delivery to the intestine is specifically regulated during hypoxia. As observed for the spot samples taken from the caudal vessel, the 5-HT concentration in blood plasma taken from the caudal vessel during the uptake experiment was not affected by hypoxia. That 5-HT concentrations in the plasma were significantly increased in + B + D + FLX fish reflects effective blockade of SERT and other transporters, as illustrated by the significant overall reductions in uptake in the atrium, ventricle, and gill in + B + D + FLX fish and as demonstrated previously (Amador and McDonald 2018b). What appears to be occurring at the mechanistic level in the present study can be observed over the long term under some circumstances. For example, a 3- to 6-day waterborne exposure of FLX of up to 100 $\mu g \cdot L^{-1}$ results in a decrease in whole-brain 5-HT concentrations in hybrid striped bass (Gaworecki and Klaine 2008), and FLX treatment for 14 days at a dose of 1.5 $\mu g \cdot g^{-1}$ per day (compared to our single dose of 50 $\mu g \cdot g^{-1}$) results in a decrease in forebrain but not hindbrain 5-HT concentrations in Siamese fighting fish (Clotfelter et al. 2007). However, some studies have measured no changes in tissue 5-HT concentrations in response to FLX alone (Mennigen et al. 2008; Sebire et al. 2015). In contrast to what our study would predict, an exposure to waterborne bupropion (38 $\mu g \cdot L^{-1}$) alone results in an increase in whole brain 5-HT concentrations in hybrid striped bass after a 6-day exposure (Sweet et al. 2016), suggesting that the findings from our very acute study using a drug combination may not translate to chronic impacts with just one of those drugs. To our knowledge, no work investigating tissue 5-HT concentrations in response to decynium-22 has been done in fish.

In contrast to our hypothesis and to the results obtained by spot sampling plasma, a significant increase in 5-HT uptake was not measured in any individual tissues or in all tissues combined in response to hypoxia exposure. In interpreting these data, we considered that it was possible that the metabolic depression likely experienced by these fish under these very low O_2 conditions (Amador et al. 2018) might have masked the enhanced uptake. Overall metabolism may have a strong influence on 5-HT dynamics, as transport of 5-HT via SERT is

both Na^+ and Cl^- dependent (Amador and McDonald 2018a) and is reliant on ion gradients produced by ATPases (reviewed by Kristensen et al. 2011) that would be slowed by reduced O_2 availability. However, a decrease in metabolism could not explain why fish that were exposed to chronic hypoxia had significantly lower plasma 5-HT in the ventral aorta than in the hepatic vein that suggested removal of 5-HT by the heart. Taking that into account, it is possible that an increase in [^{3}H]5-HT uptake, which would be measured as a higher [^{3}H] accumulation in the tissue, was not detectable because 5-HT degradation and the subsequent clearance of [^{3}H] from the tissue readily prevent [^{3}H] accumulation. Alternatively, changes in uptake at the level of particular cell types could have occurred but not have been detectable at the tissue level. For example, Stoyek et al. (2017) observed a distinct population of cells in the zebrafish atrium that appear to sequester 5-HT; it is unknown whether the 5-HT content of these cells changes with hypoxia and whether toadfish may possess similar cells in any part of the heart. Last, it is possible that 5-HT uptake from the blood is simply not involved in the protection of gas exchange during hypoxia. Instead, 5-HT uptake under normal physiological conditions may occur to control 5-HT-mediated effects on specific processes such as heart rate and contractility (Stoyek et al. 2017). Perhaps the slight reduction in heart 5-HT uptake that was actually measured could lead to tachycardia, thereby counteracting the effects of hypoxia and helping the heart to return to baseline heart rates.

There did appear to be a slightly different uptake pattern in the tissues of + B + D or + B + D + FLX fish compared to those of control fish; uptake in control fish tended to decrease with hypoxia exposure, while uptake in drug-treated fish tended to increase. BUP is known to increase O_2 consumption in mammals due to increased signaling at adrenoceptors (Liu et al. 2004, 2002), although the metabolic effects of D-22 have not been reported, and those of FLX are unclear—FLX has been found to increase metabolic rate in an invertebrate (Hird et al. 2016) but not in mammals (Gutierrez et al. 2002; Stinson et al. 1992). An increase in metabolic rate due to BUP in both treatment groups—in conflict with the likely decrease in metabolic rate due to hypoxia alone (Amador et al. 2018)—could explain the contrasting uptake patterns. Differences in metabolic rate in control vs. drug-treated fish could also explain the hypoxia-induced decrease in [^{3}H] accumulation in the bile of control fish that was absent in the drug-treated fish. The decrease in [^{3}H] accumulation in the bile of control fish likely reflects an overall slowing of metabolic rate due to hypoxia; hypoxia would likely decrease central and peripheral metabolism of 5-HT, as the activity of the 5-HT metabolic enzyme monoamine oxidase is oxygen dependent (reviewed by Gaweska and Fitzpatrick 2011). Indeed, in the mammalian brain, acute hypoxia (1–5 h) has been shown to decrease 5-HT metabolism (Davis and Carlsson 1973; Olson et al. 1983). If the [^{3}H] accumulated in the bile is at least partially composed of 5-HT metabolites—which is likely, as hypoxia did not decrease bile 5-HT in the spot sampling experiment—reduced production of these metabolites would explain the decrease in [^{3}H] in the bile of control fish exposed to hypoxia. The fact that such a decrease was not observed in treated fish could likewise be due to possible metabolic rate stimulation by BUP. Furthermore, the lack of effect of drug treatment on biliary [^{3}H] accumulation is consistent with the findings of Amador and McDonald (2018b).

Conclusions

In this study, we have shown the potential of the heart to remove circulating 5-HT from the plasma during chronic hypoxia on the basis of plasma spot sampling. We have also demonstrated the presence of high levels of 5-HT in the bile and urine of the Gulf toadfish, although more work needs to be done, for example, HPLC analysis or turnover studies during which 5-HT transport and/or degradation are inhibited, to validate these elevated concentrations. However, exposure to acute and chronic hypoxia appeared to have little effect on 5-HT uptake into toadfish tissues in this study. Thus, it is possible that the previously demonstrated SERT-mediated 5-HT uptake capacities of the heart and gill do not serve a specific purpose in the control of 5-HT during hypoxia. However, adaptive increases in uptake within the gill and heart may occur but be obscured by hypoxia-induced depression of metabolic rate or other processes, such as 5-HT degradation, occurring within the tissue. Future studies examining 5-HT uptake at oxygen levels that are low enough to elicit a cardiovascular response to hypoxia but not a reduction in metabolic rate or by

using isolated perfused hearts and/or gills of toadfish previously exposed to normoxia or chronic hypoxia could help elucidate the roles of these tissues without the potentially confounding effects of whole-animal metabolic rate changes. Furthermore, a study measuring rates of 5-HT degradation by the heart and the gill and an immunohistochemical investigation into the cellular fate of 5-HT taken up by tissues during chronic hypoxia is warranted.

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Code availability Not applicable

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