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5-Hydroxytryptamine initiates pulsatile urea excretion from perfused gills of the gulf toadfish (*Opsanus beta*)

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ABSTRACT

When stressed, toadfish become ureotelic and excrete almost all of their nitrogenous waste in 1–3 daily pulses of urea-N across the gills. Intravascular injections of 5-hydroxytyptamine (5-HT; serotonin) and analogues also elicit marked excretory pulses of urea-N from toadfish in vivo, suggesting that 5-HT release is the proximate trigger for spontaneous pulses. However it is unclear whether 5-HT is acting on the gills directly or elsewhere to cause the effect indirectly. A perfused whole gill preparation which maintained normal pressure relationships and stable vascular resistance was employed to address this question. Bolus injections into the ventral aortic perfusate of either 5-HT (1, 10 μmol kg^{-1}) or the specific 5-HT_2 receptor agonist α-methyl 5-HT (1, 10 μmol kg^{-1}) elicited rapid urea-N pulses from perfused toadfish gills. The effective doses, the post-injection delays (5.5 ± 1.3 min, range = 2–22), the percent occurrences (57–85%), and the magnitude of the induced urea-N pulses (615.4 ± 131.3 μmol-N kg^{-1}, range 66.0–2634.0), were all similar to those previously reported when these agents were injected in vivo. Bolus injections of 5-HT and α-methyl 5-HT also elicited a biphasic response in ventral aortic pressure, reflecting an initial rapid short-lived vasodilation and a subsequent longer-lasting vasoconstriction. These events were similar to those which have been recorded to occur at a greater frequency during spontaneous urea-N pulsing in vivo. Neither the urea-N pulsing nor the cardiovascular responses to 5-HT were inhibited by the 5-HT_{2A} receptor subtype blocker, ketanserin (pre-injection with 10 μmol kg^{-1} plus 33 μmol L^{-1} in the perfusate). Overall, these results provide strong support for the idea that the proximate stimulus for natural urea pulsing in vivo is 5-HT mobilization, acting directly in the gills.

1. Introduction

Unlike most other adult teleosts, the Gulf toadfish (*Opsanus beta*) expresses a full complement of ornithine–urea cycle (OUC) enzymes in its liver and white muscle (Mommsen and Walsh, 1989; Anderson and Walsh, 1995; Julsrud et al., 1998; Kong et al., 2000; Laberge et al., 2009). When subjected to crowding, confinement or several other stressors (Walsh et al., 1994; Walsh and Milligan, 1995; Hopkins et al., 1995), it transitions from ammoniotely to ureotely, a strategy which is thought to provide chemosensory crypsis for predator avoidance (Barimo and Walsh, 2006). Walsh (1997), Wood et al. (2003), and McDonald et al. (2006) have reviewed extensive earlier work on this unique strategy in the toadfish. Upon switching to ureotely, the pattern of urea-N excretion becomes pulsatile, with the whole day's urea production being vented in 1–3 short-lasting pulses, as first noticed by Walsh et al. (1990). While initial speculation focused on the gut and/or urinary systems, later experiments proved that the urea pulses originate from the gills (Wood et al., 1995; Gilmour et al., 1998), and represent pulsatile clearance of urea from the bloodstream rather than pulsatile production from the tissues (Wood et al., 1997). Considerable circumstantial evidence that the mechanism involves activation of a facilitated diffusion transporter in the gills (Gilmour et al., 1998; Wood et al., 1998; McDonald et al., 2000) culminated in the cloning of an 1814 base pair cDNA from the toadfish gill coding for a 475 amino acid protein (Walsh et al., 2000). This protein ("tUT") exhibits 60–70% homology to facilitated diffusion urea transporters of the UT-A type in higher vertebrates, and mediates greatly accelerated urea transport when expressed in *Xenopus laevis* oocytes (Walsh et al., 2000).

The mechanism behind initiation of each urea pulse has proven less tractable. A precipitous decline in circulating cortisol levels occurs before most spontaneous pulse events (Wood et al., 1997, 2001), but appears to be a permissive factor rather than a triggering factor. High cortisol, maintained experimentally, reduces the size but not the frequency of urea pulses and reduces urea transport capacity in gill baso-lateral membrane vesicles, effects mediated through glucocorticoid receptors (McDonald et al., 2004; Rodela

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et al., 2009a,b). After several attempts at establishing the triggering factor with a number of substances, including catecholamines and arginine vasotocin, that proved negative (Wood et al., 1998; Pärt et al., 1999; Wood et al., 2001), Wood et al. (2003) reported that intra-vascular injections of physiological doses of 5-hydroxytryptamine (5-HT; serotonin) elicited urea pulses of normal size with a 73% success rate. McDonald and Walsh (2004) then showed that the 5-HT_2 receptor pathway was likely involved, because a comparable pulse response was also initiated by the specific agonist α-methyl-5-hydroxytryptamine (α-methyl-5-HT) and inhibited by the specific 5-HT_{2A} antagonist, ketanserin. Furthermore, chronic treatment of toadfish with fluoxetine, a 5-HT reuptake inhibitor, raised both circulating 5-HT levels and urea excretion rates (Morando et al., 2009). The idea that 5-HT might be the proximate stimulus for urea pulsing is attractive because teleost gills exhibit an extensive serotonergic innervation, and also contain neuroepithelial cells which are rich in 5-HT (Bailly et al., 1989; Dunel-Erb et al., 1989; Bailly et al., 1992; Sundin and Nilsson, 2002; Jonz and Nurse, 2003).

Nevertheless, this idea must be treated with caution because of several *caveats*. Section of the cranial nerves to the gills altered the pattern of spontaneous pulsing, but did not abolish it (Wood et al., 2003). Furthermore, McDonald and Walsh (2004) reported that there is a finite delay of about 4 min between the intravascular injection of α-methyl-5-HT and the initiation of a pulse. This period could be a sufficient time for the serotonergic agonist to mobilize another substance (the hypothetical "true" trigger) from a systemic site (e.g. chromaffin tissue, urophysis, interrenals, juxtaglomerular cells etc.) and for the bloodstream to carry it to the gills. However, perhaps of greatest concern is the fact that Pärt et al. (1999) reported that 5-HT was one of many substances surveyed that failed to initiate urea pulsing in a perfused toadfish gill preparation. This preparation effectively isolates the gills from any influence of other factors mobilized into the systemic circulation.

The goal of the present study was to use a similar perfused toadfish gill preparation to re-examine the potential ability of 5-HT and α-methyl-5-HT to trigger urea pulsing. However, rather than using a constant concentration in the saline (the approach of Pärt et al., 1999), we employed a pre-gill bolus injection protocol similar to that which successfully elicits urea pulses in intact toadfish in vivo (Wood et al., 2003; McDonald and Walsh, 2004). The experiments also allowed us to examine actions of these agonists on vascular resistance, thereby revealing an unusual biphasic response, and clarifying the cause of post-gill pressor responses reported in a recent in vivo cardiovascular study on toadfish (McDonald et al., 2010). Overall, our results provide cogent support for the concept that 5-HT is the proximate trigger for urea pulsing in toadfish gills.

2. Materials and methods

2.1. Experimental animals

Experiments were performed in December on 50 adult Gulf toadfish (*O. beta*, Goode and Bean; 32–97 g) collected in November by roller trawl from Biscayne Bay, Florida, USA. Within 24 h, the fish were treated prophylactically with Malachite green (0.5 mg L^{-1}) plus formalin (15 mg L^{-1}). In the laboratory, they were fed frozen squid twice per week and held under "uncrowded" conditions (<5 g fish L^{-1} seawater) in 80-L aquaria supplied with individual PVC pipe shelters, and flowing seawater at 20–21 °C, 31 ppt salinity, under natural photoperiod. Approximately 1 week prior to experimentation, the fish were transferred to "crowded" conditions to induce the switch to ureotely: 6 toadfish were housed together in 6-L plastic tubs (i.e. ~50 g fish L^{-1} seawater), again supplied with shelters, and flowing seawater. The fish were not fed during this "crowding" treatment.

2.2. Gill perfusion

The perfused gill preparation was a modification of the method of Pärt et al. (1999) for toadfish. The principal difference was that the head was left attached to the body, similar to the preparation of Wood et al. (1978) for trout, such that the systemic resistance downstream of the gills was maintained. The backpressure in the dorsal aorta is important in minimizing gill resistance and maintaining normal perfusion patterns (Wood, 1974; Wood et al., 1978).

Toadfish from the crowded treatments were injected via the caudal vessels with 2500 i.u. sodium heparin (Sigma-Aldrich, St. Louis, MO, USA) in 0.5 mL saline at 10 min prior to sacrifice. They were then rapidly euthanized in MS-222 (Argent Laboratories, Redmond, WA, USA; 2.5 g L^{-1} neutralized with NaOH), and the spinal cord was severed, taking care not to cut the dorsal aorta. Heat-flared PE 90 tubing (Clay-Adams, Sparks, MD, USA) was tied into the ventral aorta with silk suture for perfusion inflow, and the ventricular outflow was cannulated with a U-shaped loop of PE160 tubing to collect the effluent perfusate. A ventilation Y-tube was sewn to the roof of the mouth so as to direct water flow over each set of gills. The ventilation reservoir was a Nalgene™ bottle holding approximately 200 mL of seawater, which was mounted horizontally, and into which the toadfish's head was sealed using a sheet of medium burr dental dam affixed posterior to the opercular openings but anterior to the pectoral fins. The body was cradled at the same level inside a PVC pipe. Water was removed from the bottom of reservoir by a column pump (Biorad, Hercules, CA, USA), and returned through the ventilation Y-tube and over the gills at a rate of 200 mL min^{-1}. An air-stone in the reservoir ensured atmospheric PO_2, and temperature was maintained at 20–21 °C. The exact volume of the seawater in the ventilation reservoir was measured at the end of the experiment.

The perfusate was delivered to the ventral aorta through one channel of a Minipuls 3 peristaltic pump (Gilson, Middleton, WI, USA) at a nominal rate of 1.0 mL 100 g^{-1} min^{-1}; the exact rate was determined gravimetrically. A T-port in the PE 90 tubing just proximal to the point of cannulation provided a site for bolus injections. A second T-port was connected to a pressure transducer (Statham P23BB, Statham Instruments, Oxnard, CA, USA) interfaced to a transducer amplifier (Harvard Apparatus, Holliston, MA, USA), and writing on a chart recorder. Pressure was measured in cm H_2O, with calibration against a column of water.

The perfusate was a physiological saline containing (in mmol L^{-1}) 148.0 NaCl, 2.70 Na_2HPO_4, 10.0 $NaHCO_3$, 2.60 KCl, 1.24 $MgSO_4$, 1.26 $CaCl_2$, 5.0 glucose, and 10.0 urea (= 20 mmol L^{-1} urea-N). Immediately prior to use, the saline was spiked with 10 i.u. mL^{-1} of sodium heparin and 50,000–100,000 cpm mL^{-1} of ^{14}[C]urea (0.05–0.10 μCi L^{-1}; New England Nuclear, Boston, MA, USA), and equilibrated with 0.3% CO_2/99.7% O_2. Bovine serum albumin fraction V (BSA, Sigma; 20 g L^{-1}) was then added. The saline was stirred thoroughly while the same gas passed through the airspace of the flask (bubbling was avoided to prevent protein flocculation), the pH was adjusted to 7.8, and finally the perfusate was filtered through a 0.22 μm filter (Millipore, Billerica, MA, USA) prior to use.

The preparation was perfused for 8 min to allow stabilization and radioisotopic equilibration, and then sampling was begun (0 min). In the standard protocol, the first 20 min served as a control period to determine the baseline rates of urea-N excretion then a single bolus injection of agonist (in 100 μl saline per 100 g total body mass) was administered. Each preparation was used for only one injection. Water samples (1 mL) for [^{14}C]urea assay were drawn from the ventilation reservoir at 2-min intervals for 60 min so as to track the appearance of urea-N in the external water, and perfusion pressure was measured throughout. At the end of the experiment, the inflow catheter was removed from the ventral aorta with sutures intact,

and then perfused in situ to record the resistance of the delivery system.

The following agents were administered as bolus injections, each in a separate preparation (N = 6–9 per treatment): saline, 1 μmol kg^{-1} 5-HT, 10 μmol kg^{-1} 5-HT, 1 μmol kg^{-1} α-methyl-5-HT, and10 μmol kg^{-1} α-methyl-5-HT. An additional treatment was carried out in which the 5-HT_{2A} receptor antagonist, ketanserin (10 μmol kg^{-1}), was injected intraperitoneally into the live toadfish at 1 h prior to the experiment, and also added to the perfusion saline at a concentration of 33 μmol L^{-1} so as to ensure effective blockade; a bolus injection of 10 μmol kg^{-1} 5-HT was tested in these preparations. The doses of 5-HT, a-methyl 5-HT and ketanserin used in the present study were as described in previous studies (Wood et al., 2003; McDonald and Walsh, 2004). All drugs were obtained from Sigma-Aldrich (St. Louis, MO, USA).

[^{14}C]urea radioactivity was measured on an LS 180-1 liquid scintillation counter (Beckman-Coulter, Indianapolis, IN, U.S.A.), using 1-mL seawater samples or 0.05-mL perfusate samples (plus 0.95 mL seawater) added to 3 mL Ecolume fluor (MP Biomedicals, Solon, OH, USA). Tests showed that quench was constant.

2.3. Calculations and statistics

The vascular resistance of the preparation (in cm H_2O 100 g min mL^{-1}) was calculated as the true perfusion pressure (in cm H_2O, corrected for the perfusion pressure of the delivery system) divided by the perfusion flow rate (in mL 100 g^{-1} min^{-1}). As the measured flow rates were almost exactly 1.0 mL 100 g^{-1} min^{-1}(Table 1), the vascular resistances (in cm H_2O 100 g min mL^{-1}) were numerically equal to the true perfusion pressures (in cm H_2O).

Urea fluxes (in μmol-N kg^{-1} h^{-1}) were calculated from the rate of appearance of [^{14}C]urea radioactivity (cpm mL^{-1} h^{-1}) in the external seawater reservoir, factored by measured specific activity (μmol-N cpm^{-1}) of the perfusion medium, measured reservoir volume (mL), time (h), and weight (kg). The rate of [^{14}C]urea radioactivity excretion over the first 20 min (prior to the bolus injection) was used to establish the baseline rate, and was extrapolated to 60 min to predict the total excretion at the baseline rate over 1 h. This value was then subtracted from the actual cumulative excretion at 60 min so as to calculate the size of any urea-N pulses which occurred, as illustrated by the examples in Fig. 3.

Urea flux rates (in μmol-N kg^{-1} h^{-1}) were converted to estimated gill urea permeability values (in cm s^{-1}) as described by Pärt et al. (1999) using the perfusate-to-seawater concentration gradient (20 μmol-N L^{-1}) and a gill area of 1920 cm^2 kg^{-1}, based on the measurements of Hughes and Grey (1972).

Data have been expressed as means ± 1 standard error (N), where N represents the number of preparations. Multiple comparisons were made by 1-way ANOVA followed by Fisher's LSD test. Where necessary, data were appropriately transformed (ln, square root) to achieve normality of distribution and homogeneity of variance. Probabilities associated with percent occurrence data were analyzed by Fisher's exact probability test.

3. Results

3.1. Vascular resistance

Vascular resistance of the preparations was stable throughout the 60-min perfusion period in the control group, which were injected with only saline at 20 min (Table 1). A similar stability of baseline resistance was seen in the groups injected with the two doses of 5-HT and α-methyl 5-HT. The overall vascular resistance was 20.35 ± 1.50 cm H_2O 100 g min mL^{-1} (N = 37), representing a mean ventral aortic perfusion pressure of about 20.3 cm H_2O, because mean perfusion flow rate was very close to the nominal rate of 1 mL 100 g^{-1} min^{-1} in all groups (Table 1).

However, a very different pattern was seen in the ketanserin group (10 μmol kg^{-1} pre-treatment, 1 h prior to sacrifice), where initial resistance and perfusion pressure were only 40–60% of the values in all the other groups, a significant difference ($P<0.01$) (Table 1). Resistance thereafter rose rapidly, more than doubling in the first 20 min, and continuing to increase more gradually through until 60 min; note that ketanserin (33 μmol L^{-1}) was also present in the perfusion medium throughout this period, so the effect cannot be explained by washout of the drug.

Typical responses in perfusion pressure to agonist injections are illustrated in Fig. 1, and mean vascular resistance changes tabulated in Fig. 2. Bolus injections of saline caused only a small depressor response, generally lasting less than 1 min (Figs. 1A and 2); there was no significant pressor response. However both 5-HT and α-methyl-5-HT, at both doses (1 and 10 μmol kg^{-1}) induced a biphasic response, first decreasing pressure to a greater extent and/or duration than saline, and thereafter increasing it above the baseline level (Figs. 1B, D, E and 2). The latter vasoconstrictory response was of much longer duration than the vasodilatory response, often lasting more than 20 min post-injection. With 5-HT both the depressor and pressor responses were dose-dependent, but with α-methyl 5-HT this was only true for the pressor effect (Fig. 2).

Table 1
Perfusion flow rates, vascular resistances, and ventral aortic perfusion pressures in perfused toadfish gill preparations in various treatment groups. Means ± 1 SEM (N).

Treatment	Perfusion flow rate (mL 100 g^{-1} min^{-1})	Vascular resistance = Ventral aortic perfusion pressure (cm H_2O 100 g min mL^{-1}) (cm H_2O)		
		0 min	20 min	60 min
Saline control (N = 8)	1.067 ± .088	19.32 ± 3.20	17.67 ± 2.72	17.80 ± 2.75
5-HT (1 μmol kg^{-1}) (N = 6)	1.005 ± .071	19.36 ± 1.37	19.18 ± 0.95	18.45 ± 1.29
5-HT (10 μmol kg^{-1}) (N = 9)	0.987 ± .007	24.69 ± 1.85	24.75 ± 2.27	25.45 ± 1.78
5-HT (10 μmol kg^{-1}) + ketanserin (N = 6)	0.996 ± .005	11.43 ± 1.27*	26.96 ± 7.71	34.06 ± 6.44§
α-Methyl 5-HT (1 μmol kg^{-1}) (N = 7)	1.005 ± .009	18.29 ± 3.69	17.06 ± 3.67	18.33 ± 2.68
α-Methyl 5-HT (10 μmol kg^{-1}) (N = 6)	0.980 ± .007	25.48 ± 5.50	23.87 ± 5.70	29.35 ± 4.91

* $P<0.05$ relative to all other 0 min values.
§ $P<0.05$ relative to 0 min value in this treatment.
There were no other significant differences ($P>0.05$).
Note: All treatments (except 5-HT + ketanserin) were identical until 20 min, when different agonists were bolus injected. In the ketanserin treatment, 10 μmol kg^{-1} of ketanserin was administered in vivo at 1 h prior to sacrifice, and was also present in the perfusion medium at 33 μmol L^{-1}.

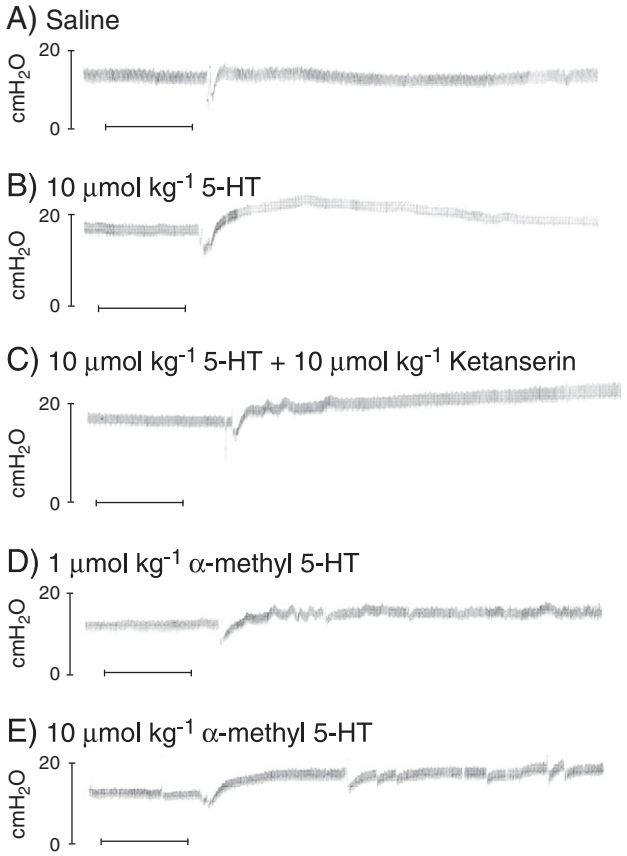

Fig. 1. Original recordings of ventral aortic perfusion pressure in 5 different perfused toadfish gill preparations. Typical responses to bolus injections of (A) saline (1 mL kg^{-1}); (B) 10 μmol kg^{-1} 5-HT; (C) 10 μmol kg^{-1} 5-HT in a ketanserin-treated preparation; (D) 1 μmol kg^{-1} alpha-methyl 5-HT; and, (E) 10 μmol kg^{-1} alpha-methyl 5-HT, are shown. Note that only a depressor response occurred with saline, but a biphasic response with alpha-methyl 5-HT and 5-HT. Note also the persistence of these responses in the ketanserin-treated preparation. Scale bar = 1 min.

However, at the same dose, there were no significant differences in potency of the two agonists. Ketanserin had no significant effects on either the vasodilatory or vasoconstrictory responses to 5-HT injections (Figs. 1C and 2).

3.2. Urea-N excretion

Low baseline urea-N excretion rates were recorded in the first 20 min in 29 preparations (Table 2). Rates which averaged more than 18-fold higher were observed in 13 preparations during this same period; these were interpreted as preparations which were spontaneously pulsing.

Bolus injections of either 5-HT or α-methyl 5-HT, at both doses (1 and 10 μmol kg^{-1}) elicited discrete pulses of greatly elevated urea-N excretion in most preparations, as illustrated in Fig. 3A and B. The mean delay between injection and start of the urea-N pulse was 5.5 ± 1.3 min (N = 23; range = 2–22 min), and more than half (13) of these preparations pulsed within 2 min post-injection. With one exception, injections of saline did not elicit urea-N pulses (Fig. 3C). In one additional preparation, illustrated in Fig. 3D, an apparent spontaneous urea pulse started at 8 min.

The percent occurrence of urea-N pulses after injections of either 5-HT or α-methyl-5-HT was 57–83% (N = 6–9 per treatment), relative to 12% (1 out of 8) in the saline controls, and did not appear to be related to the dose of the agonist (Fig. 4A). The probabilities that these pulses were caused by the agonist, and not by the saline vehicle injection, were all in the range of 80–90% by Fisher's exact probability test. Pulse size appeared to be dose-dependent for both agonists, though none of the differences among agonists or doses were statistically significant (Fig. 4B). The mean rate of urea excretion-N and the gill urea-N permeability during the period of these agonist-induced pulses was 15.5 fold greater than baseline rates, and very similar to the rates recorded in those preparations which were spontaneously pulsing (Table 2). The ketanserin treatment had no effect on either the percent occurrence or the magnitude of 5-HT induced urea-N pulses.

In preparations which exhibited low background rates of urea-N excretion until 20 min, the overall success rate of any agonist injection causing urea-N pulses was 73% (N = 26) versus only 44% (N = 9) in preparations which appeared to be already spontaneously pulsing in the first 20 min; the probability that this difference was real was 88%. However there was no difference in pulse size or in vascular resistance. When preparations that pulsed in response to agonist injections were compared to those that did not, there were again no significant differences in vascular resistance, or in either the mean vasodilatory or vasoconstrictory responses of the two groups. There were also no significant relationships between pulse size and

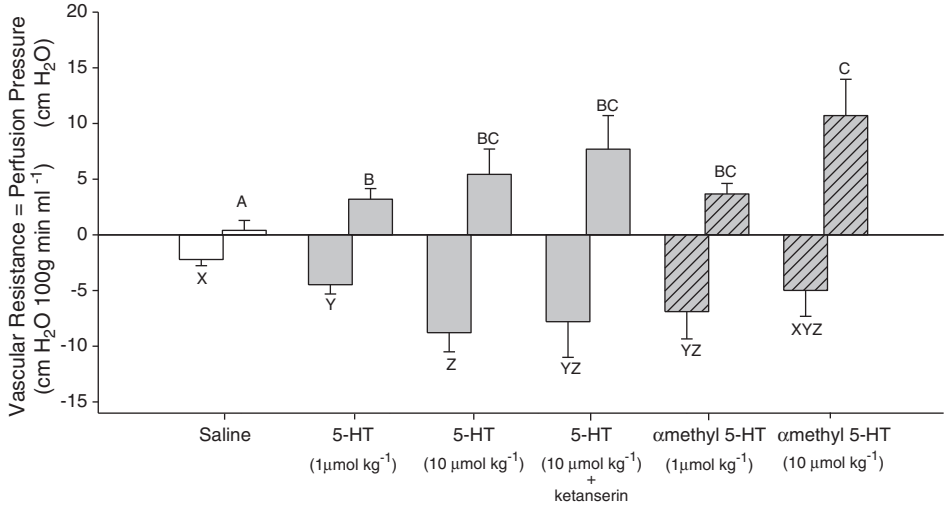

Fig. 2. Changes in vascular resistance associated with bolus injections of agonists in perfused toadfish gill preparations. The negative bars represent the initial vasodilatory responses, and the positive bars represent the following more prolonged vasoconstrictory responses. Peak responses are tabulated. Means ± 1 SEM (N = 6–9). Within each response type, means sharing the same letter are not significantly different from one another (P > 0.05). In the ketanserin treatment, 10 μmol kg^{-1} of ketanserin was administered in vivo at 1 h prior to sacrifice, and was also present in the perfusion medium at 33 μmol L^{-1}.

Table 2
Urea-N excretion rates and gill urea-N permeabilities in perfused toadfish gill preparations. Means ± 1 SEM (N).

	Urea-N excretion rate (μmol-N kg^{-1} h^{-1})	Urea-N permeability (cm s$^{-1}\times10^{-7}$)	
		This study	Pärt et al. (1999)
Baseline	72.8 ± 14.5 (29)	5.26 ± 1.04 (29)	4.16 ± 0.40 (16)
Spontaneously pulsing	1340.5 ± 278.1 (13)	96.96 ± 20.11 (13)	68.22 ± 26.56 (4)
Pulsing induced by agonists	1127.8 ± 210.8 (29)	81.49 ± 15.23 (29)	–

the magnitudes of either the depressor or pressor responses. Overall, the size of the urea-N pulses induced by 5-HT or α-methyl 5-HT from the perfused preparations averaged 615.4 ± 131.3 μmol-N kg^{-1} (N = 23; range 66.0–2634.0 μmol-N kg^{-1}). In most cases (e.g. Fig. 3A, B, D), the pulse appeared to be coming to an end by 60 min, but there were a few exceptions, so overall pulse size was likely underestimated.

4. Discussion

The key finding of the present study is that 5-HT itself, and the specific 5-HT$_2$ agonist α-methyl 5-HT, both elicit rapid urea-N pulses from perfused toadfish gills. The effective doses (1 and 10 μmol kg^{-1}) are the same as those which are effective in vivo, the post-injection delays (~5 min) are similar, as are the percent occurrences (57–85%), and the magnitude of the induced urea-N pulses (~615 μmol-N kg^{-1}), and the elevation (~15.5 fold) of urea-N excretion above background rates (Wood et al., 2003; McDonald and Walsh, 2004). This provides strong support for the idea that the proximate stimulus for urea pulsing in vivo is 5-HT mobilization at the gill. The pulse-inducing effect of exogenous 5-HT injections in vivo is duplicated in this in vitro preparation, and is not due to the mobilization of another substance from a systemic site which is then carried to the gills by the bloodstream.

Baseline urea-N permeability and its degree of elevation (~18-fold) in spontaneously pulsing preparations were both very similar to the values reported by Pärt et al. (1999) in an earlier perfused toadfish gill study (Table 2). The frequency of occurrence of these spontaneously pulsing events was recorded as 4 out of 20 (20%) by Pärt et al. (1999) whereas the ratio was 13 out of 42 (31%) in the present study; the two ratios are not significantly different. However Pärt et al. (1999) reported that 5-HT was ineffective in causing urea-N pulses. The difference between the present positive results and the negative findings of Pärt et al. (1999) is most likely due to the different modes of 5-HT administration, though it may also be due to differences in the nature of the gill preparations.

In the present study, bolus injections (1 and 10 μmol kg^{-1}) were administered into the ventral aortic perfusion line just before it entered the gills, whereas Pärt et al. (1999) used constant concentrations of 10^{-7} M–10^{-6} M, which is within the circulating range for 5-HT in blood plasma of catheterized, confined toadfish (Wood et al., 2003). The bolus injections of the present study would likely have raised the perfusate 5-HT concentration passing through the gills by 2–4 orders of magnitude above this, into the range which might occur at nerve synapses. The other difference is the presence of the systemic resistance to provide back-pressure to the gills; in the isolated head preparation of Pärt et al. (1999), there was no back pressure component. Using a comparable perfusion flow, Pärt et al. (1999) reported a gill vascular resistance of about

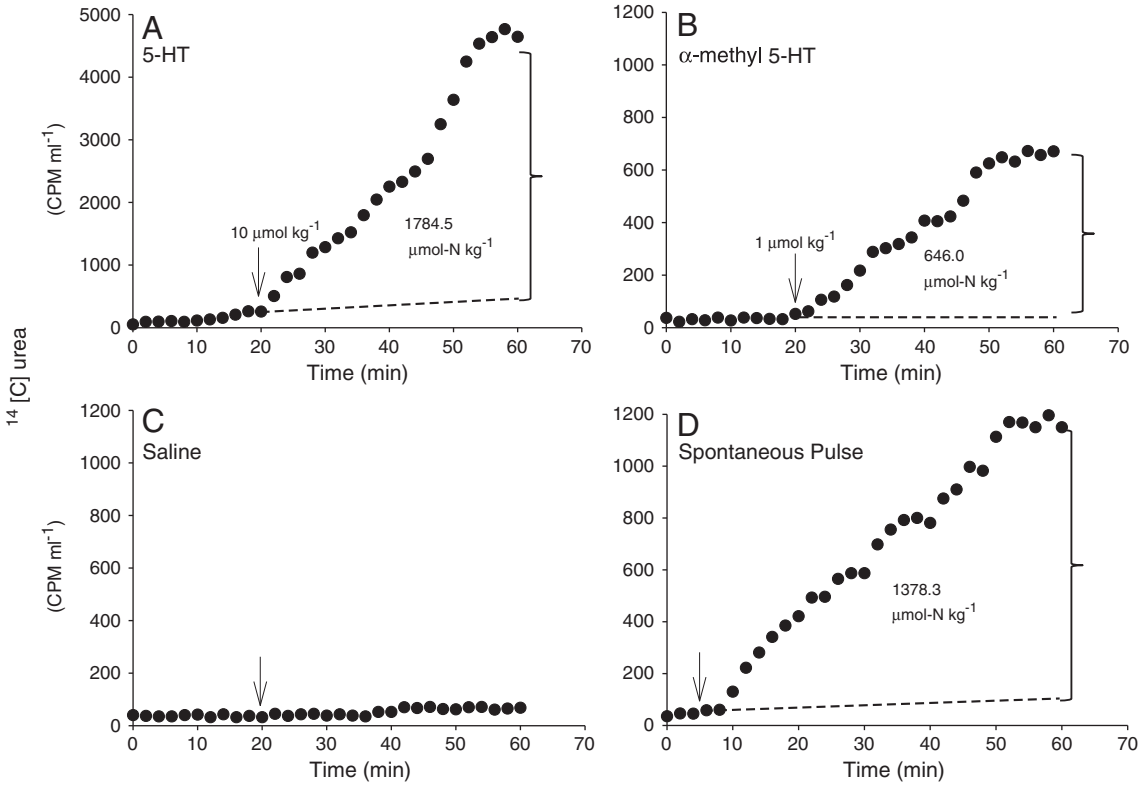

Fig. 3. Typical [^{14}C]urea-N pulse responses of 4 different perfused toadfish gill preparations injected with (A) 10 μmol kg^{-1} 5-HT, (B) 1 μmol kg^{-1} alpha-methyl 5-HT, and (C) saline only (1 mL kg^{-1}) at 20 min (arrows). Panel (D) shows an apparent spontaneous pulse starting without injection at 8 min (arrow). The dotted lines represent extrapolation of the original pre-pulse base-line rates of urea-N excretion to 60 min. The difference between the cumulative [^{14}C]urea-N excretion at this time and the extrapolated baseline value was used to calculate the size of the urea-N pulse, as shown.

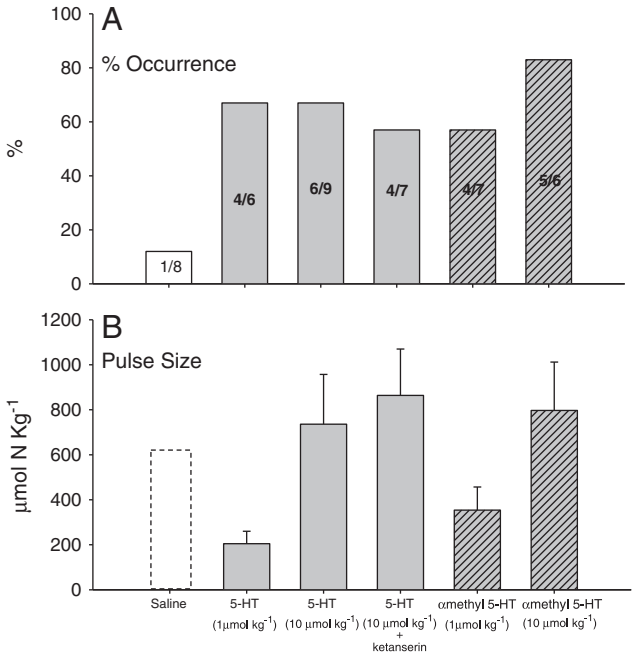

Fig. 4. (A) Percent occurrence and (B) mean size (±1 SEM; N numbers as shown in panel A) of urea-N pulses exhibited by perfused toadfish gill preparations in response to the experimental treatments noted in panel (B). The single pulse occurring after saline injection is shown with a dashed line. In the ketanserin treatment, 10 µmol kg^{-1} of ketanserin was administered in vivo at 1 h prior to sacrifice, and was also present in the perfusion medium at 33 µmol L^{-1}.

23 cm H_2O 100 g min mL^{-1}, whereas we recorded a similar value (Table 1) for the whole preparation (gill resistance plus systemic resistance). It seems likely that the systemic resistance would be at least half of the total (Wood, 1974; Wood et al., 1978), so the gill resistance of our preparation was probably only about 50% of that of Pärt et al. (1999), thanks to the influence of the dorsal aortic back-pressure. This may have created a more effective perfusion, opening up areas of the gill not perfused by Pärt et al. (1999).

We suggest that during natural pulsing events, 5-HT acts as a trigger, being mobilized from serotonergic nerve-endings, and/or 5-HT-rich neuroepithelial cells in the gills (Bailly et al., 1989; Dunel-Erb et al., 1989; Bailly et al., 1992; Sundin and Nilsson, 2002; Jonz and Nurse, 2003). The 5-HT surge would activate tUT in gill epithelial cells by post-transcriptional modification; Walsh et al. (2000) identified several potential phosphorylation and glycosylation sites within the tUT amino acid sequence. Transcriptional activation probably does not occur, because tUT mRNA levels appear to be at their lowest at the time of natural pulses, and then increase again in a cortisol- dependent fashion in the period between pulses (Rodela et al., 2011). It is difficult to reconcile this scheme with the data reported by Wood et al. (2003) showing that bilateral section of all branchial branches of cranial nerves IX (glossopharyngeal) and X (vagus) did not prevent spontaneous urea-pulsing in toadfish, though it did modify the pattern, doubling the frequency and halving the pulse magnitude. However, in that study, one important set of cranial nerves, the branches of VII (facial), could not be sectioned because they provide essential innervation to the breathing muscles. It is also now clear that, in addition to centrally-derived innervation, there is a network of intrinsic serotonergic neurons in the gills, and both this network and the 5-HT rich neuro-epithelial cells survive in explant culture after degeneration of extrinsic nerve fibers (Jonz and Nurse, 2003). Future studies should examine potential direct effects of 5-HT in gill epithelial cells, and the potential for autonomous activity in the intrinsic nerve net.

α-Methyl 5-HT was equally effective to 5-HT in causing urea-N pulsing in perfused toadfish gills (Fig. 4), in agreement with the in vivo findings of McDonald and Walsh (2004). Based on the action of α-methyl 5-HT in the mammalian system, these data implicate the 5-HT_2 receptor family, of which there are three subtypes 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C}, in the control of urea excretion in toadfish. To our knowledge, α-methyl 5-HT does not target any non-serotonergic mammalian receptors. Ketanserin, an antagonist of mammalian 5-HT_2 receptors, targeting the mammalian 5-HT_{2A} receptor subtype with the highest affinity, did not block the pulse-inducing action of 5-HT. At first glance, this may appear to disagree with the data of McDonald and Walsh (2004) showing that ketanserin inhibited the pulse-inducing action of α-methyl 5-HT in vivo in a dose-dependent fashion. However, in the present study, ketanserin was tested against the natural agonist 5-HT and not against α-methyl 5-HT (Barnes and Sharp, 1999). Not all α-methyl 5-HT effects are blocked by ketanserin in toadfish (see McDonald et al., 2010, as discussed below) and an even wider range of serotonergic receptor types can be activated by 5-HT (Barnes and Sharp, 1999; Hoyer et al., 2002). Perhaps these data suggest that there is redundancy in the system such that 5-HT mobilization elicits urea-N pulses through several receptor types, and that blockade of only one of them is not sufficient to prevent this action. Along these lines, the mammalian UT-A2, to which tUT is most similar on the molecular level (Walsh et al., 2000), is controlled by two second messengers, cAMP and intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) (Potter et al., 2006). In mammals, all 5-HT_2 receptor subtypes are linked to increases in ($[Ca^{2+}]_i$) but are not associated with change in cAMP (Barnes and Sharp, 1999). We believe our data suggest that the regulation of tUT may be similar to UT-A2, i.e., sensitive to both cAMP and ($[Ca^{2+}]_i$), and perhaps another 5-HT receptor subtype that is insensitive to ketanserin but linked to cAMP, such as the 5-HT_4, 5-HT_6 or 5-HT_7, is involved. Incidentally, both the 5-HT_4 (LR Medeiros and MD McDonald, unpublished) and the 5-HT_7 receptor (M.D. McDonald and M. Grosell, unpublished) have been described on a pharmacological level in toadfish, and on the molecular level in amphibians (*Xenopus laevis*, 5-HT_7, Genbank Accession #*NM_001085784*) and invertebrates (*Aplysia californica*, 5-HT_4, *HM187584.1*).

Since the pharmacological work by McDonald and Walsh (2004) suggesting the involvement of the 5-HT_{2A} receptor in the regulation of toadfish urea excretion, this receptor subtype has been sequenced in toadfish (*FJ611960.2*) and shows 59% similarity to the mammalian receptor on the amino acid level, with 100% conservation in the amino acid residues associated with ligand binding (Shapiro et al., 2000; E.M. Mager, L.R. Medeiros, A.P. Lange, M.D. McDonald, unpublished). While the specificity of the toadfish 5-HT_{2A} receptor for ketanserin has not yet been directly tested, the high molecular conservation of this receptor within these very important residues does support pharmacological specificity. Nevertheless, ketanserin has also been shown to bind to mammalian α-adrenergic and histamine receptors with a moderate binding affinity (Leysen et al., 1980). While two studies (Wood et al., 2003; McDonald and Walsh, 2004) support the role of 5-HT in regulating pulsatile urea excretion and the potential role of catecholamines in this regulation have been ruled out in previous studies (Perry et al., 1998; Wood et al., 1998; Pärt et al., 1999), the ability of ketanserin to bind to α-adrenergic and histamine receptors could have implications for the vascular response observed in response to ketanserin treatment (see below).

While 5-HT and α-methyl 5-HT both exerted strong effects on the vascular resistance of the preparation (Fig. 2), there appeared to be no quantitative association between these effects and urea-N pulsing. Nevertheless they occurred at more or less the same time. Gilmour et al. (1998) carried out long-term monitoring of caudal artery (= dorsal aortic, i.e., post-gill) blood pressure in toadfish in vivo, and could detect no specific association between blood pressure and spontaneous urea-N pulsing. Nevertheless, these workers reported the occurrence of spontaneous biphasic blood pressure events very

similar to the biphasic responses seen in the ventral aorta with bolus injections of 5-HT and α-methyl 5-HT in the present study (compare their Fig. 5 with our Fig. 1). Furthermore, Gilmour et al. (1998) concluded that spontaneous pressure events occurred with a significantly higher frequency during periods of natural urea-N pulsing. While the evidence is circumstantial, it points to an involvement of 5-HT surges in both events.

A later study examined the caudal arterial pressure response to intra-venous injections of α-methyl 5-HT ($1\ \mu mol\ kg^{-1}$) in intact toadfish, and detected only a pressor response at this post-gill site (McDonald et al., 2010). Interestingly, this pressor response to the 5-HT_2 receptor agonist was not blocked by ketanserin, the more specific $5HT_{2A}$ inhibitor, so the pattern parallels the current results (Fig. 2). Notably however, the pre-treatment with ketanserin markedly lowered vascular resistance of the perfused preparation, an effect that has been observed in mammalian heart and pulmonary circulation and has been attributed to 5-HT_{2A} receptors (Hood et al., 1998; Delaney et al., 2011). However, ketanserin has also been shown to antagonize α-adrenergic receptors (Leysen et al., 1980; Van Nueten et al., 1981), albeit with a lower affinity than for 5-HT_{2A} receptors. Therefore α-adrenergic blockade cannot be ruled out as a reason for the low peripheral resistance observed initially in toadfish pre-injected with ketanserin. Furthermore, ketanserin's moderate binding affinity for histamine receptors (Leysen et al., 1980), where histamine has been shown to be involved in vasodilation in the tropical cichlid, *Oreochromis niloticus* (Okafor and Oduleye, 1986), could explain the later increase in toadfish vascular resistance with subsequent ketanserin perfusions.

Given the nature of the current preparation in which the ventral aortic pressure changes are measured at constant flow at a site upstream from two resistances in series (branchial and systemic), they are not confounded by changes in cardiac output. However we cannot conclusively separate branchial from systemic effects. Nevertheless, based on the time course we speculate that the initial rapid vasodilatory response to 5-HT and α-methyl 5-HT occurs in the gills whereas the later more long-lasting vasoconstrictory response occurs in the systemic circulation, or else in both resistances. The branchial vasodilation plus the systemic vasoconstriction would thereby both contribute to the post-gill pressor responses recorded by McDonald et al. (2010) in toadfish in vivo. These vascular responses appear to be unusual, because most studies on other teleosts have shown that 5-HT constricts the gills and dilates the systemic circuit (reviewed by Sundin and Nilsson, 2002; McDonald et al., 2010). Yet again, *O. beta* is proving to be a most unusual teleost.

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