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Dogmas and controversies in the handling of nitrogenous wastes: 5-HT_2-like receptors are involved in triggering pulsatile urea excretion in the gulf toadfish, *Opsanus beta*

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Summary

When injected arterially, serotonin (5-hydroxytryptamine; 5-HT) has been shown to elicit naturally sized urea pulse events in the gulf toadfish, *Opsanus beta*. The goal of the present study was to determine which 5-HT receptor(s) was involved in mediating this serotonergic stimulation of the pulsatile excretion mechanism. Toadfish were surgically implanted with caudal arterial catheters and intraperitoneal catheters and injected with either 8-OH-DPAT (1 μmol kg^{-1}), a selective 5-HT_{1A} receptor agonist, α-methyl-5-HT (1 μmol kg^{-1}), a 5-HT_2 receptor agonist, or ketanserin, a 5-HT_2 receptor antagonist (0.01, 0.1, 1 and 10 μmol kg^{-1}) plus α-methyl-5-HT. 8-OH-DPAT injection did not mediate an increase in urea excretion, ruling out the involvement of 5-HT_{1A} receptors in pulsatile excretion. However, within 5 min, α-methyl-5-HT injection caused an increase in the excretion of urea in >95% (*N*=27) of the fish injected, with an average pulse size of 652±102 μmol N kg^{-1} (*N*=26). With α-methyl-5-HT injection there was no corresponding increase in ammonia or [^{3}H]PEG 4000 permeability. Urea pulses elicited by α-methyl-5-HT were inhibited in a dose-dependent fashion by the 5-HT_2 receptor antagonist ketanserin, which at low doses caused a significant inhibition of pulse size and at higher doses significantly inhibited the occurrence of pulsatile excretion altogether. However, neither 8-OH-DPAT nor α-methyl 5-HT injection had an effect on plasma cortisol or plasma urea concentrations. These findings suggest the involvement of a 5-HT_2-like receptor in the regulation of pulsatile urea excretion.

Key words: serotonin, ketanserin, serotonin receptor, α-methyl-5-HT, 8-OH-DPAT.

Introduction

Unraveling the mystery surrounding the pulsatile urea excretory mechanism in the toadfish gill has been the focus of research for over a decade. The gulf toadfish, unusual in that it has a fully functional ornithine–urea cycle (for others, see Saha and Ratha, 1987, 1998; Randall et al., 1989; Wood et al., 1989, 1994), has the ability to deviate from the obligate ammoniotelism typical of most teleost fish and excrete predominantly urea when stressed by crowding, confinement, air or ammonia exposure (Walsh et al., 1990, 1994; Walsh and Milligan, 1995). What makes the toadfish unique is that the excretion of urea, of which >90% occurs across the gills, is not continuous, as is the excretion of ammonia. Instead, urea is excreted in distinct pulses, once or twice a day, lasting 0.5–3 h in duration, suggesting the periodic activation or insertion of a selective urea transporter (Wood et al., 1995, 1997, 1998). An important finding in this research was the molecular characterization of a facilitated diffusion urea transporter (tUT; Smith et al., 1998; Walsh et al., 2000) in the toadfish gill that shows a 70% homology at the amino acid level to mammalian UT-A2 facilitated diffusion mechanisms. The expression of tUT mRNA has been found to be largely invariant over the pulse cycle (Walsh et al., 2000), suggesting a shorter-term, non-genomic mechanism of tUT activation.

Linked to this periodic increase in urea permeability across the gill is a dramatic decrease in plasma cortisol levels, suggesting that circulating cortisol may be an important regulator of pulsatile urea excretion (Hopkins et al., 1995; Wood et al., 1997, 2001). When ureotelic, toadfish maintain plasma cortisol concentrations that are typical for chronically (but moderately) stressed teleosts (reviewed by Mommsen et al., 1999). However, 2–4 h preceding a natural urea pulse event, plasma cortisol levels fall steadily and then rise rapidly thereafter (Wood et al., 1997, 2001). Since plasma cortisol levels will also decrease without the occurrence of a natural pulse, the decline in cortisol concentrations is not believed to be the direct trigger to pulsatile urea excretion (Wood et al., 2001). While the decline in cortisol may be permissive to pulsatile excretion, a recent study has suggested that the drop in cortisol does not have to take place in order for pulses to occur (M. D. McDonald, C. M. Wood, M. Grosell and P. J.

Walsh, unpublished data). Indeed, continuous infusion with cortisol in an attempt to prevent a pre-pulse decline in levels had no effect on the frequency of urea pulses, although the infusion did cause a significant reduction in pulse size. These recent results suggest perhaps a role for cortisol in the regulation of the number of transporters, i.e. through the regulation of transcription as described in mammalian UT transporters (Knepper et al., 1975; Naruse et al., 1997; Peng et al., 2002) or perhaps through non-genomic pathways. However, cortisol may not directly be involved in the regulation of the activation of the transport mechanism.

Most recently, Wood et al. (2003) outlined the possibility of serotonin (5-hydroxytryptamine; 5-HT) as the trigger for the pulsatile mechanism since arterial injections of this substance result in pulses of natural size. Serotonin is implicated in a variety of psychological and physiological roles in mammals and is intimately associated with the mammalian stress response, namely the regulation of the hypothalamic–pituitary–adrenal (HPA) axis, resulting in an elevation in circulating cortisol (reviewed by Chaouloff, 1993; Carrasco and Van De Kar, 2003). Potentially relevant to pulsatile excretion, 5-HT has also been shown to regulate the hypothalamic–pituitary–interrenal axis (HPI), the teleost homologue of the mammalian HPA axis (Winberg et al., 1997; Overli et al., 1999; Höglund et al., 2002). Reciprocally, central 5-HT synthesis and/or release is under complex control by glucocorticoids in mammals (reviewed by Chaouloff, 1993; Carrasco and Van De Kar, 2003). In mammals, 5-HT_{1A} and 5-HT_{2A} receptors have been attributed to mediating serotonergic activation of the HPA axis (reviewed by Barnes and Sharp, 1999), and evidence suggests that a 5-HT_{1A}-like receptor is present (Yamaguchi and Brenner, 1997) and exerts the same function in teleosts (Winberg et al., 1997; Höglund et al., 2002).

Therefore, the goal of this study was to determine the 5-HT receptors involved in the regulation of the pulsatile urea excretion mechanism of the gulf toadfish. Since circulating cortisol and urea pulse events are strongly correlated in toadfish, the hypothesis tested was that either 5-HT_{1A} or 5-HT_2 5-HT receptors mediate 5-HT-triggered urea pulse events. To test this hypothesis, toadfish were treated with agonists and antagonists specific for these types of receptors and the pattern of urea excretion was characterized. In addition, the cortisol response to these pharmacological agents was monitored.

Materials and methods

Experimental animals

Gulf toadfish [*Opsanus beta* Goode and Bean; 0.074±0.003 kg (*N*=50); range, 0.044–0.140 kg] were captured with a roller trawl by commercial shrimpers in Biscayne Bay, Florida in the winter of 2002–2003. The toadfish were held in an outdoor tank at the shrimpers' holding facility with running sea water (ambient seasonal conditions) for no longer than 24 h following capture, then transferred to the laboratory where they were held for up to one month. Fish were treated with a dose of malachite green (final concentration 0.05 mg l^{-1}) in formalin (15 mg l^{-1}) (AquaVet, Hayward, CA, USA) on the day of transfer to the laboratory in order to prevent infection by the ciliate *Cryptocaryon irritans* (Stoskopf, 1993). Initially, the fish were kept in 50-liter glass aquaria with flowing, aerated seawater. Fish were maintained in this relatively crowded environment (>10 fish per tank) in order to initiate a switch to ureotelism (Walsh et al., 1994). The water temperature was 24–26°C. Fish were fed weekly with squid up until the time of surgery.

Experimental protocol

As outlined by Wood et al. (1997) and McDonald et al. (2000), caudal artery catheterizations were performed on fish anaesthetized with MS-222 (0.5 g l^{-1}; Sigma-Aldrich, St Louis, MO, USA) and wrapped with wet towels. Intraperitoneal (i.p.) catheters (Clay-Adams PE50) filled with toadfish saline (Walsh, 1987) were inserted through a small ventral incision and threaded approximately 4 cm inside the body cavity. The wound was treated with oxytetracycline powder in order to prevent infection and the catheter sutured securely with 3-0 silk at the site of exit. An additional suture secured the catheter just anterior to the arterial catheter incision and two more secured it along the arterial PE 160 sleeve. The fish were left to recover undisturbed in individual shielded flux chambers with a PVC pipe shelter for 24 h, at which time the patency of the arterial catheters was confirmed. The functioning arterial and i.p. catheters were then left outside the flux chamber attached to a syringe, so as not to disturb the fish a second time by searching for the catheters within the chamber. The fish were left overnight (for a total of 36 h of recovery), after which time the water flow to the flux chamber was stopped. Without disturbing the fish, the water level quickly dropped to an exact volume mark of 1.35–2.0 liters through a small hole in the flux chamber, and vigorous aeration maintained thorough mixing and the oxygen partial pressure (P_{O_2}) close to air saturation. A water sample (5 ml) was taken for the measurement of initial urea and ammonia concentration and [^{3}H]PEG 4000 counts. Thereafter, water samples were taken every hour until the injection of the first pharmacological agent (see below), after which water samples were taken approximately every 30 min (unless otherwise stated) for the remainder of the experiment.

Pharmacological experiments

Pharmacological experiments were performed in order to determine the 5-HT receptors involved in triggering the pulsatile urea excretion mechanism of the gulf toadfish. The pharmacological agents used in the present study included the selective 5-HT_{1A} receptor agonist 8-OH-DPAT [8-hydroxy-2-(di-n-pro-pylamino) tetralin hydrobromide; Sigma-Aldrich], the 5-HT_2 receptor agonist α-methyl-5-HT (Sigma-Aldrich) and the 5-HT_2 receptor antagonist ketanserin (ketanserin tartrate salt; Sigma-Aldrich). Eight different experimental series were performed. In Series 1 and Series 2, fish were injected (1 μmol ml^{-1} saline kg^{-1} fish) with either 8-OH-DPAT (*N*=6) or α-methyl-5-HT (*N*=27), respectively, *via* the

arterial catheter followed by 3 ml kg^{-1} toadfish saline. This dose was calculated to yield circulating concentrations of 3×10^{-6} mol l^{-1}, which is close to circulating serotonin levels measured in toadfish (10^{-7}–10^{-6} mol l^{-1}) and identical to the dose of serotonin used on toadfish in a recent study (Wood et al., 2003). In a separate experimental series (Series 3), the possibility of a non-specific branchial permeability increase in response to α-methyl-5-HT injection was examined by injecting a dose of 50 μCi kg^{-1} body mass of [^{3}H]PEG 4000 (Perkin Elmer, Wellesley, MA, USA) *via* the caudal arterial catheter followed by an additional 3 ml kg^{-1} of saline (*N*=8). Toadfish were then left overnight to allow for equilibration of the [^{3}H]PEG 4000 throughout the extracellular space as described by McDonald et al. (2000). In Series 4, the time for α-methyl-5-HT to elicit a urea pulse was measured (*N*=6). In this series, a timer was started immediately following injection of the first toadfish and water samples were taken precisely every five minutes for the next 50 min. At the same time, each remaining fish was injected and the injection time noted. An estimate of the time it took for each individual fish to pulse was then determined. In four separate experimental series (Series 5, *N*=4; Series 6, *N*=5; Series 7, *N*=5; Series 8, *N*=7), four different doses of the 5-HT$_2$ receptor antagonist ketanserin (0.01, 0.1, 1 and 10 μmol ml^{-1} kg^{-1}) were injected *via* an i.p. catheter in 45% (w/v) HBC (2-hydroxypropyl-β-cyclodextrin, a non-toxic solubilizer; Sigma-Aldrich) one hour before the arterial injection of α-methyl-5-HT. Due to the necessity of a solubilizing agent, i.p. injection of ketanserin was considered to be a more suitable method of treatment.

Analytical techniques and calculations

Urea concentrations in blood and water were measured using the diacetyl monoxime method of Rahmatullah and Boyde (1980), with appropriate adjustments of reagent strength for the different urea concentration ranges in water and blood plasma. Ammonia concentrations in the water were measured by the indophenol blue method (Ivancic and Degobbis, 1984). Plasma cortisol concentrations were measured using a commercial [^{125}I] radioimmunoassay kit (ICN Biomedicals Inc., Costa Mesa, CA, USA) with standards diluted to the same protein range as toadfish plasma. For measurements of [^{3}H]PEG 4000, water samples (2 ml) were added to 10 ml of Ecolume fluor (ICN Biomedicals Inc.) and analyzed by scintillation counting on a TM Analytic 6895 BetaTrac counter.

The majority of urea excretion is *via* the gills (>90%), the kidney of toadfish contributing only a small percentage (<10%) (McDonald et al., 2000). In addition, the permeability of the skin to urea is extremely low [$5.07\pm0.56\times10^{-7}$ cm s^{-1} (*N*=8); Pärt et al., 1999]. The excretion (*E*; μmol kg^{-1}) of any substance (X) was calculated from the increase in concentration of the substance in the water $[\Delta X]_w$ during a pulse corrected for fish body mass (*m*) and calculated as:

$$E_X = \frac{[\Delta X]_w \times V_f}{m}, \quad (1)$$

where V_f is the volume of water surrounding the fish. A pulse was identified as a sudden increase in urea appearance in the surrounding water of at least 40 μmol N kg^{-1}. Fish that pulsed within the 4 h period prior to agonist injection were excluded from the study, due to the refractory period associated with pulsatile excretion as described by Wood et al. (1997).

Statistics

Data are reported as means ± 1 S.E.M. (*N*=number of fish). In the case when only two means are compared, the significance of differences between means was evaluated using Student's unpaired two-tailed *t*-test (*P*<0.05; Nemenyi et al., 1977). When looking at the significance of differences between a treatment group over time, a one-way, repeated measures analysis of variance (ANOVA) with time as the main factor was used and followed by a Bonferroni correction for multiple comparisons. When looking at the significance of differences between two treatment groups over time, a two-way repeated measures ANOVA with time and treatment group as the main factors was used and followed by a Holm–Sidak test for multiple sample comparisons.

Results

A urea pulse event was not elicited by the injection of the 5-HT$_{1A}$ receptor agonist 8-OH-DPAT (Fig. 1A). While fish injected with 8-OH-DPAT did experience a significant elevation of plasma cortisol concentrations over time (ANOVA, *P*<0.001), this increase was also measured in the injection control (Fig. 1B). Plasma urea concentrations were relatively constant throughout the experiment (Table 1).

In contrast to 8-OH-DPAT, the 5-HT$_2$ receptor agonist α-methyl-5-HT caused a dramatic increase in the excretion of urea, eliciting urea pulse events in >95% (*N*=27) of all fish injected, with a mean pulse size of 652±102 μmol N kg^{-1} (*N*=26; Fig. 2A). There was no corresponding elevation in the excretion of ammonia or [^{3}H]PEG 4000, indicating that the increase in the permeability of the gill was specific for urea (Fig. 2B,C). Remarkably, α-methyl-5-HT mediated its effect within 5 min [4.25±0.41 min (*N*=8)] of injection, with a pulse size within that time frame of 517±121 μmol N kg^{-1} (*N*=8; Fig. 3). However, the constraints of the sampling protocol most likely resulted in an overestimation of this time, as in two cases a urea pulse event was measured from individuals after <3 min following α-methyl-5-HT injection. The mean duration of one urea pulse was 6.75±2.63 min (*N*=8), with seven of eight fish completing the pulse within 5 min (i.e. one increasing increment of urea appearance) and one of eight taking 25 min to complete one pulse (i.e. five consecutive increasing increments of urea appearance). Within the first 30 min of α-methyl-5-HT injection, five of eight fish pulsed a second time, with a mean pulse size of 468±256 μmol N kg^{-1} (*N*=5) and a mean duration of 7.0±1.2 min (*N*=5; Fig. 3).

Treatment with the 5-HT$_2$ receptor antagonist ketanserin caused a significant, dose-dependent inhibition of pulse events elicited by α-methyl-5-HT (Fig. 4A). Intraperitoneal injection of the vehicle (HBC) alone had no effect on the potency or the

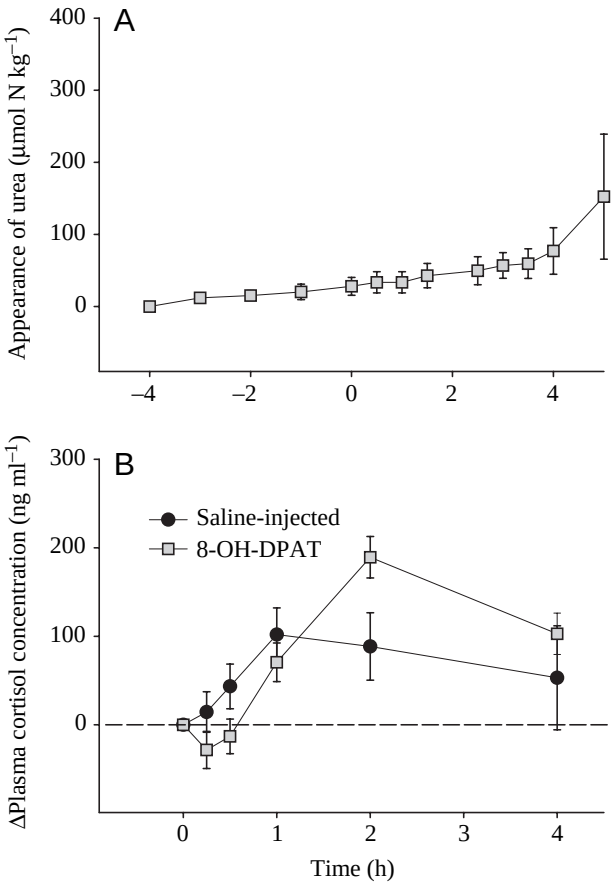

Fig. 1. (A) Injection of 8-OH-DPAT (*N*=6) had no effect on the appearance of urea in the water. (B) Fish injected with 8-OH-DPAT did show a significant increase in plasma cortisol levels; however, this increase was also measured in the saline-injected controls (*N*=16; ANOVA, *P*<0.001). All values are means ± 1 S.E.M.

pulse size elicited by α-methyl-5-HT (*N*=4), thus values were combined with those measured from fish injected with the agonist alone. Ketanserin not only caused a significant, dose-dependent decrease in the size of urea pulses in those fish that did respond to α-methyl-5-HT injection (Fig. 4B) but also caused a dose-dependent decrease in the percentage of fish that pulsed upon injection with α-methyl-5-HT (Fig. 4C). Urea pulse size was sensitive to inhibition by the antagonist and was inhibited at concentrations of ketanserin (0.01 μmol ml^{-1} kg^{-1}) that were two orders of magnitude less than that of α-methyl-5-HT (Fig. 4B). While pre-treatment with ketanserin at doses lower than the agonist (0.01 or 0.1 μmol ml^{-1} kg^{-1} antagonist *versus* 1 μmol ml^{-1} kg^{-1} agonist) resulted in a significant reduction in urea pulse size, the number of fish that responded to α-methyl-5-HT by pulsing, i.e. the effectiveness of α-methyl-5-HT to elicit a urea pulse, did not change (Fig. 4C). However, the effectiveness of α-methyl-5-HT to elicit urea pulses was significantly inhibited at doses of ketanserin that were greater than or equal (1 and 10 μmol ml^{-1} kg^{-1}) to the agonist (Fig. 4C). Despite pulsatile excretion of urea, plasma urea concentrations were relatively constant throughout all experimental treatments (Table 1).

Similar to fish treated with 8-OH-DPAT, fish injected with α-methyl-5-HT did experience a significant elevation of plasma cortisol concentrations over time that was not statistically different from the injection control, suggesting that the blood sampling protocol alone caused the rise in cortisol (data not shown). Furthermore, pre-treatment with ketanserin at any dose had no effect on plasma cortisol concentrations compared with α-methyl-5-HT alone or the injection control (data not shown).

Discussion

The present investigation clearly demonstrates that the urea pulse events triggered by 5-HT (Wood et al., 2003) are mediated by a 5-HT_2-like receptor, since urea pulses were elicited by the injection of α-methyl-5-HT (a 5-HT_2 receptor agonist) and not by 8-OH-DPAT (a specific 5-HT_{1A} receptor agonist). In addition, urea pulses mediated by the 5-HT_2 receptor agonist were inhibited in a dose-dependent fashion by the 5-HT_2 receptor antagonist ketanserin, which at low doses caused a significant inhibition of pulse size and at high doses significantly inhibited the occurrence of pulsatile excretion altogether.

In mammals, the 5-HT_2 receptor family currently consists of three receptor subtypes, 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}, which

Table 1. *Plasma urea concentrations (mmol l^{-1}) of toadfish prior to pharmacological treatment (*t*=0) and at consecutive time points post-injection*

			α-methyl-5-HT + ketanserin			
Time (h)	8-OH-DPAT	α-methyl-5-HT	0.01	0.1	1	10
0	6.4±0.3 (6)	8.0±0.8 (10)	7.3±1.0 (4)	5.4±0.4 (5)	6.3±0.3 (5)	4.6±0.3 (7)
0.25	6.2±0.5 (6)	–	8.0±1.4 (4)	5.3±0.5 (5)	6.3±0.3 (5)	4.6±0.3 (7)
0.5	6.4±0.4 (6)	8.4±0.8 (10)	7.9±1.5 (4)	5.3±0.6 (5)	6.2±0.3 (5)	4.6±0.3 (7)
1	6.3±0.5 (6)	8.7±0.9 (10)	8.1±1.5 (4)	5.3±0.7 (5)	6.1±0.3 (5)	4.5±0.3 (6)
2	6.5±0.5 (5)	8.9±1.0 (10)	8.3±1.5 (4)	5.2±0.6 (5)	6.3±0.3 (5)	4.9±0.1 (6)
4	6.8±0.4 (6)	8.3±0.8 (10)	8.4±1.6 (4)	5.4±0.6 (5)	6.4±0.4 (5)	4.7±0.3 (7)

All values are means ± 1 S.E.M. (*N*=number of fish).

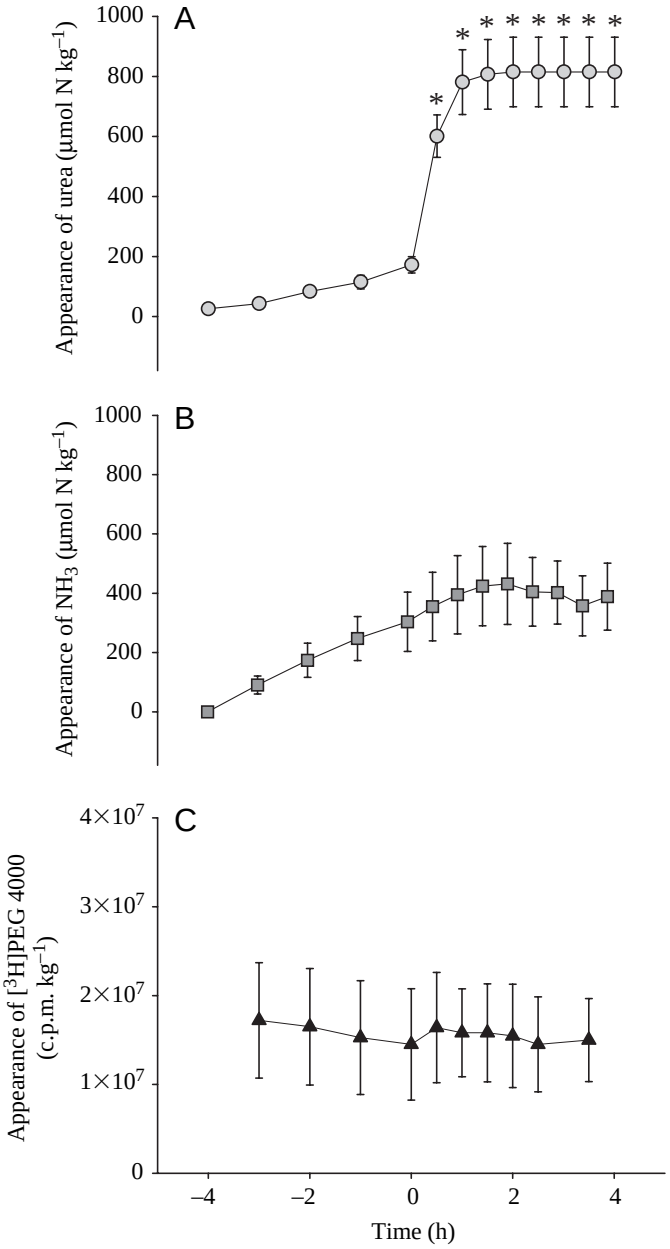

Fig. 2. Injection of α-methyl-5-HT caused a significant increase in (A) the appearance of urea in the water (N=27) with no corresponding increase in (B) the appearance of NH_3 (N=6) or (C) the appearance of [^{3}H]PEG 4000 (N=8). All values are means ± 1 S.E.M.; *P<0.05, significantly different from value at t=0.

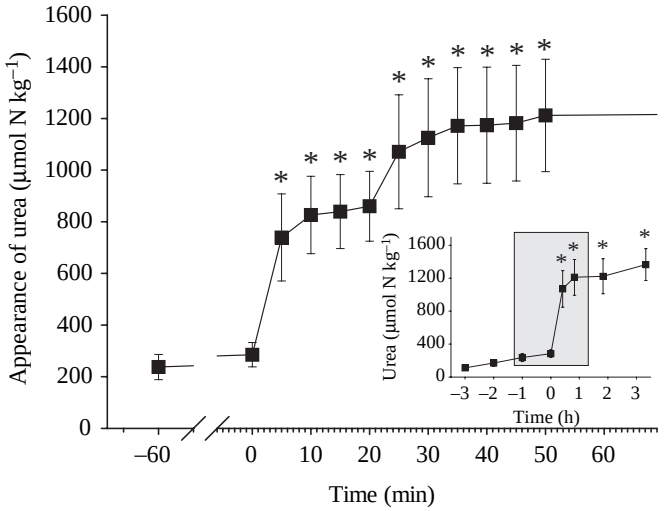

Fig. 3. Water samples taken every 5 min from a separate group of α-methyl-5-HT-injected fish, demonstrating that the agonist injection results in a significant and pulsatile increase in the excretion of urea within 5 min. Inset shows coarser time scale for comparison with Fig. 2. All values are means ± 1 S.E.M. (N=6); *P<0.05, significantly different from value at t=0.

are similar in terms of their molecular structure, pharmacology and signal transduction pathways (reviewed by Barnes and Sharp, 1999). All genes in the 5-HT$_2$ receptor family couple positively to phopholipase C and mobilize intracellular calcium and inositol phosphates (Hoyer et al., 1994). In addition, 5-HT$_2$ receptors have a relatively low affinity for 5-HT, which may explain why an injection of 5-HT at the same dose as α-methyl-5-HT is less effective at eliciting urea pulses (Wood et al., 2003).

In mammals, α-methyl-5-HT shows a selectivity between the three different 5-HT$_2$ receptor subtypes, with pEC$_{50}$ values of 8.4, 7.3 and 6.1 corresponding to a selectivity of 5-HT$_{2B}$>5-HT$_{2C}$>5-HT$_{2A}$ (Baxter et al., 1995; reviewed by Barnes and Sharp, 1999). Ketanserin also differentiates between the three different subtypes, with pK_i values of 8.9, 7.0 and 5.4 corresponding to a selectivity of 5-HT$_{2A}$>5-HT$_{2C}$>5-HT$_{2B}$ (Baxter et al., 1995; reviewed by Barnes and Sharp, 1999). This indicates that ketanserin is almost 100-fold more and over 1000-fold more selective for 5-HT$_{2A}$ receptors than for either 5-HT$_{2C}$ or 5-HT$_{2B}$ receptors, respectively. In addition, 5-HT$_{2A}$ is almost 1000-fold more sensitive to ketanserin than to α-methyl-5-HT, 5-HT$_{2C}$ is approximately equal in sensitivity to the two compounds, and 5-HT$_{2B}$ is 1000-fold less sensitive to ketanserin than to α-methyl-5-HT. In the present study, ketanserin first caused a significant inhibition at a dose 100-fold lower than that of the agonist, corresponding to an IC$_{50}$ of approximately 0.0095 μmol l^{-1}. Based on the affinities of the 5-HT$_2$ family of receptors for the agonist and antagonist, an antagonist IC$_{50}$ that is 100 times less than the effective concentration of the agonist suggests that 5-HT$_{2A}$ receptors are involved in the mediation of pulsatile urea excretion. However, the specific involvement of the 5-HT$_{2A}$ receptor subtype is yet to be positively identified.

The rapid action of α-methyl-5-HT to elicit a urea pulse event suggests that the 5-HT$_2$ receptor could be in close proximity to the urea transporter(s) involved in pulsatile urea excretion. A recent study gives evidence against central nervous system (CNS) activation of individual urea transporters, as there is no effect of bilateral surgical sectioning of cranial nerves IX (glossopharyngeal) and X (vagus) on pulse size (Wood et al., 2003). That being the case, a co-localization

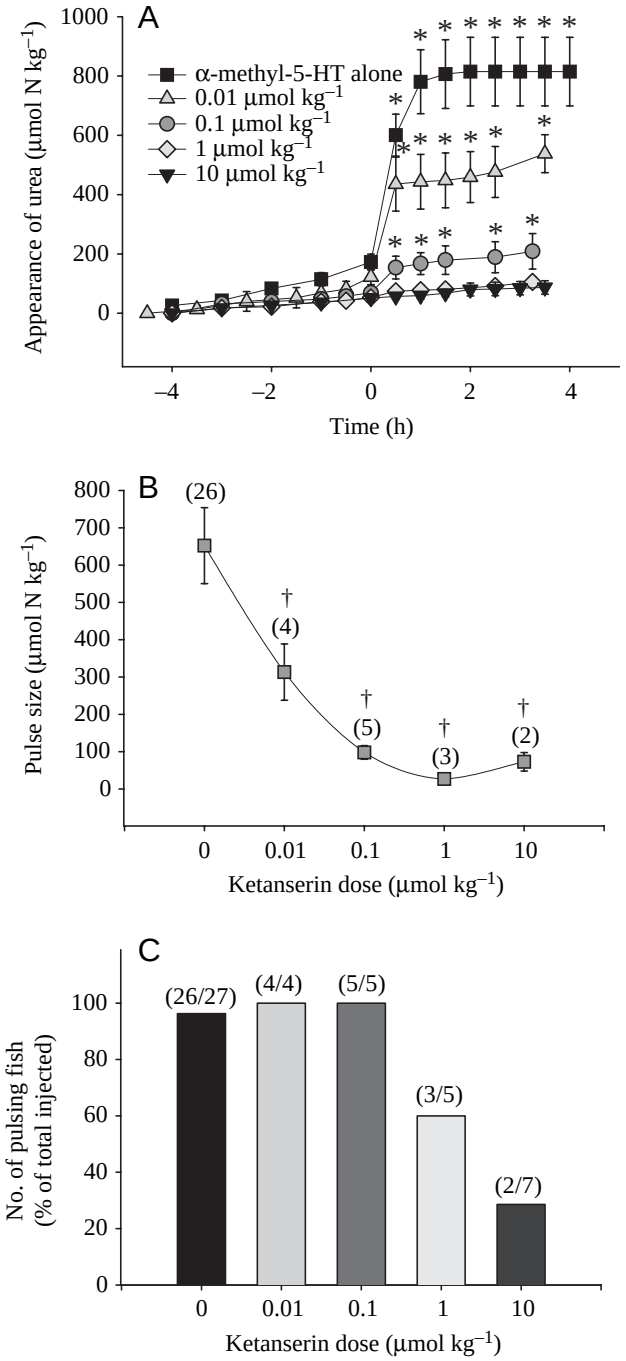

Fig. 4. (A) Pre-injection with ketanserin caused a significant inhibition of the α-methyl-5-HT-mediated effect. (B) Ketanserin mediated a dose-dependent decrease in pulse size. (C) The percentage of fish that pulsed following agonist injection. All values are means ± 1 S.E.M.; *P<0.05, significantly different from value at t=0; †P<0.05 compared with α-methyl-5HT injection alone.

of tUT and 5-HT_2 could potentially result in the direct activation of tUT through 5-HT_2 receptor-mediated phosphorylation. Sequence analysis of tUT shows two potential phosphorylation sites within a much longer C-terminal sequence that is unique to tUT and has been suggested to be related to the rapid upregulation of urea transport during a urea pulse (Walsh et al., 2000). Correspondingly, ketanserin could then be inhibiting the 5-HT_2-mediated phosphorylation of proximal tUTs, reducing the number of activated transporters and subsequently causing a reduction in pulse size. Theoretically, increasing the dose of ketanserin could result in a more pronounced reduction in activated transporters until a threshold is achieved when so few transporters are activated that a urea pulse can no longer be detected.

In addition to 5-HT_2 receptors and tUT being in close proximity, it is also possible that the site of 5-HT release is close to or within the gill. As discussed above, central mediation does not appear to be involved. However, there are several 5-HT storage sites located outside the CNS and even within the gill of teleost fish that could potentially be sources of 5-HT for triggering urea pulsing *in vivo*. These include neuroepithelial cells (NECs) and neurons (Dunel-Erb et al., 1982, 1989; Bailly et al., 1989, 1992; Jonz and Nurse, 2003) located within the gill itself as well as the posterior cardinal vein that runs adjacent to the kidney (Fritsche et al., 1993). While the data of the present study suggest the involvement of 5-HT_2 receptors by introducing a specific concentration of agonist and antagonist into the entire circulation, the relevant concentration of 5-HT necessary to elicit urea pulses *in vivo* may be local gill levels, which are difficult to predict.

A previous study by Wood et al. (1997) described sharp drops in plasma urea concentrations in association with natural pulse events, which return to pre-pulse concentrations within 4–6 h. In the present study, plasma urea concentrations show no relationship with α-methyl-5-HT-induced urea pulses as they do not change over the course of the experiment. However, the findings of the present study describe an activation of branchial urea excretion upon introducing α-methyl-5-HT into the systemic circulation. It is possible that by doing so, tissue urea transporters are also activated, thereby allowing an even more rapid equilibration of tissue and plasma, as even under resting conditions there is excellent equilibration of urea between the water compartments of plasma, liver and white muscle (Wood et al., 1997). This might not be the case during natural pulsatile excretion, when there could likely be a more localized, branchial release of 5-HT. The variation observed in initial plasma urea concentrations between the different groups of fish is consistent with previous observations (reviewed by Wood et al., 2003).

There are extensive data lending support to an excitatory influence of central serotonergic systems upon the HPA axis in mammals and the HPI axis in fish (see reviews by Chaouloff, 1993; Carrasco and Van De Kar, 2003). Depending on the dose and status of the fish, 8-OH-DPAT has been shown to cause both increases and decreases in circulating cortisol levels, suggesting the presence of 5-HT_{1A}-like receptors as mediators of HPI activity in teleosts (Winberg et al., 1997; Höglund et al., 2002). In the present study, 8-OH-DPAT injection did not result in an increase in plasma cortisol concentrations that was significantly different from injection controls. Interestingly, central 5-HT_{1A} sensitivity is under the permissive control of

glucocorticoids in mammals, and high cortisol levels decrease the sensitivity but not the B_{max} of these receptors (Laaris et al., 1997; Czyrak et al., 2002). The high circulating cortisol levels typical of cannulated toadfish in the present study (200–400 ng ml^{-1}; Wood et al., 1997, 2001; M. D. McDonald, C. M. Wood, M. Grosell and P. J. Walsh, unpublished data) could have served to decrease the sensitivity of the 5-HT$_{1A}$ receptor, making it difficult to further stimulate the HPI axis in these fish. However, the lack of a substantial cortisol response mediated by 5-HT$_{1A}$ or 5-HT$_2$ receptor agonists could also suggest that these receptors are not involved in the regulation of the HPI axis in toadfish. Indeed, in amphibians, 5-HT$_4$ receptors and not 5-HT$_{1A}$ or 5-HT$_2$ receptors mediate the direct stimulatory effect on glucocorticoid release (Idres et al., 1991).

Thus, 5-HT$_2$ receptors appear to be involved in mediating the activation of the urea pulse, but they are probably not directly responsible for the changes in plasma cortisol observed in toadfish around the time of a pulse. However, a strong correlation still exists between cortisol fluctuations and urea pulses in toadfish under normal, resting conditions (Wood et al., 1997, 2001). While the activation of the urea transporter mediated by 5-HT$_2$ receptors is a relatively fast event (<5 min), the natural pulsatile process is rather slow; cortisol levels drop 2–4 h prior to a urea pulse and then rise within 2 h thereafter (Wood et al., 1997, 2001). Perhaps through the sensitization/desensitization of 5-HT$_2$ receptors, fluctuating plasma cortisol concentrations during the natural pulse cycle are involved in mediating pulsatile urea excretion. In fact, cortisol-infused toadfish show a significant 70% decrease in the size of urea pulses (M. D. McDonald, C. M. Wood, M. Grosell and P. J. Walsh, unpublished data), lending support to the theory that high circulating cortisol concentrations may have a desensitizing effect on 5-HT$_2$ receptors.

Notably, 5-HT also stimulates arginine vasopressin (AVP; antidiuretic hormone) secretion *via* 5-HT$_2$ receptors, whereas 5-HT$_{1A}$ receptors appear not to be involved (Jorgensen et al., 2003). In mammals, AVP is an important stimulator of UT-A-facilitated urea transport by mediating acute, cAMP-dependent changes in the activity of membrane-bound UT-A proteins as well as through a gradual recruitment of transporters to the membrane from intracellular pools (Grantham and Burg, 1966; Star et al., 1988; Inoue et al., 1999; reviewed by Smith and Rousselet, 2001). With respect to the toadfish, arterial injection of arginine vasotocin (AVT; the teleost homologue of AVP) does cause a urea pulse event to occur, suggesting the involvement of this hormone in pulsatile urea excretion (Perry et al., 1998; Wood et al., 2001). However, these urea pulses are at most 10% the size of natural pulses, and occur only at supraphysiological levels of AVT (10^{-10}–10^{-9} mol l^{-1}). As mammalian AVP is also a regulator of the HPA axis, an interaction between teleostean AVT, cortisol and 5-HT$_2$ receptors in the regulation of pulsatile urea excretion cannot entirely be ruled out and will be directly investigated in the future.

Interestingly, both 5-HT and α-methyl-5-HT have been shown to cause a rapid and pronounced constriction of gill blood vessels, resulting in an increase in branchial vascular resistance and a reduction in arterial oxygen pressure (Sundin et al., 1998). It is not believed that the increase in branchial vascular resistance associated with α-methyl-5-HT injection results in a non-selective increase in branchial permeability, as simultaneous increases in branchial urea, ammonia and PEG 4000 excretion would have been evident in the present study. While the physiological significance of 5-HT on branchial resistance and gas transfer is unknown (Sundin et al., 1998), it has been suggested that 5-HT may be involved in ventilation (Fritsche et al., 1992), acid–base balance (Thomas et al., 1979) or environmental sensory systems (Dunel-Erb et al., 1982; Bailly et al., 1989). In theory, the factors that cause variations in any one of these systems could also be potential cues for pulsatile urea excretion in toadfish under natural conditions.

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