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The toadfish serotonin 2A (5-HT_{2A}) receptor: molecular characterization and its potential role in urea excretion

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ABSTRACT

Based on early pharmacological work, the serotonin 2A (5-HT_{2A}) receptor subtype is believed to be involved in the regulation of toadfish pulsatile urea excretion. The goal of the following study was to characterize the toadfish 5-HT_{2A} receptor at a molecular level, to determine the tissues in which this receptor is predominantly expressed and to further investigate the pharmacological specificity of toadfish pulsatile urea excretion by examining the effect of ketanserin, a 5-HT_{2A} receptor antagonist, on resting rates of pulsatile urea excretion. The full-length toadfish 5-HT_{2A} receptor encodes a 496 amino acid sequence and shares 57–80% sequence identity to 5-HT_{2A} receptors of other organisms, with 100% conservation among important ligand-binding residues. Toadfish 5-HT_{2A} receptor mRNA expression was highest in the swim bladder and gonad, followed by the whole brain. All other tissues tested (esophagus, stomach, anterior intestine, posterior intestine, rectum, liver, kidney, heart, muscle and gill) had mRNA expression levels that were significantly less than whole brain. Toadfish 5-HT_{2A} receptor mRNA expression within the brain was highest in the hindbrain, telencephalon and midbrain/diencephalon regions. Treatment with the 5-HT_{2A} receptor antagonist, ketanserin, resulted in a significant decrease in the pulsatile component of spontaneous urea excretion due to a reduction in urea pulse size with no significant change in pulse frequency. These results lend further support for the 5-HT_{2A} receptor in the regulation of pulsatile urea excretion in toadfish.

1. Introduction

In mammals, the serotonin (5-hydroxytryptamine; 5-HT) 2A (5-HT_{2A}) receptor subtype is well-described on a molecular and pharmacological level and has been shown to be involved in a range of physiological and behavioral processes, such as vascular smooth muscle contraction, hormone secretion, Parkinson's disease, anorexia and bulimia nervosa, schizophrenia, and major depressive disorder (reviewed by Nagatomo et al., 2004; Kaye et al., 2005; Fox et al., 2008; Lohoff, 2010; Ebdrup et al., 2011). It is one of three receptors in the 5-HT_2 receptor family, the other two being the 5-HT_{2B} and 5-HT_{2C} receptors, and all three are similar in their signal transduction pathways in that they activate phospholipase C which leads to an increase in inositol phosphate and intracellular [Ca^{2+}] (reviewed by Barnes and Sharp, 1999).

The 5-HT_{2A} receptor has been characterized on a molecular basis in zebrafish, *Danio rerio* (GenBank Accession # XM_683178) and tilapia, *Oreochromis niloticus* (# XM_003449546.1). While no pharmacological or physiological work has been done on this receptor in these particular fish species, there is ample evidence for 5-HT_{2A}-like receptor involvement in a variety of physiological responses in teleost fish based on what is known about the pharmacology of the mammalian form of this receptor. In particular, many studies in fish have noted a sensitivity to the mammalian 5-HT_{2A} receptor antagonist, ketanserin. For example, based on the *in vivo* or *in vitro* effects of ketanserin, the 5-HT_{2A} receptor has been shown to be responsible for mediating the 5-HT-induced stimulation of gonadotropin releasing hormone release in red seabream (*Pagrus major*; Senthikumaran et al., 2001) and gonadotropin release in goldfish, *Carassius auratus* (Somoza et al., 1988; Somoza and Peter, 1991), and Atlantic croaker, *Micropogonias undulatus* (Khan and Thomas, 1992). It also appears to be involved in intestinal contraction in rainbow trout (Burka et al., 1989) and it may be involved in modulating the electric signal from the electric fish, *Brachyhypopomus pinnicaudatus* (Allee et al., 2008). Most recently, it appears that the 5-HT_{2A} receptor may regulate oxygen sensing in the amphibious mangrove rivulus, *Kryptolebias marmoratus* (formerly the mangrove killifish, *Rivulus marmoratus*) (Regan et al., 2011) and hypoxia-induced bradycardia in the Gulf toadfish, *Opsanus beta* (McDonald et al., 2010).

In the Gulf toadfish, a 5-HT_{2A}-like receptor is also believed to be involved in the regulation of urea excretion (McDonald and Walsh, 2004). Toadfish are unique among teleost fish in that they have a fully functional ornithine–urea cycle which enables them to be facultatively ureotelic when exposed to environmental stressors. While urea production has been shown to be constant, the excretion of

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urea into the water is periodic or pulsatile, occurring once or twice a day on average (Wood et al., 1997; 1998). Urea passes across the gill *via* the toadfish urea transporter (tUT), a facilitated diffusion urea transport mechanism similar to mammalian UT-A2 urea transporters (Walsh et al., 2000). Both the stress hormone cortisol and the neurotransmitter 5-HT are believed to be involved in the periodic activation or insertion of tUT into the gill membrane (Wood et al., 1997, 2001, 2003; McDonald et al., 2004, 2009; Rodela et al., 2009), where low circulating cortisol levels are believed to be necessary to allow for the activation of tUT by 5-HT.

McDonald and Walsh (2004) found that arterial injections of the mammalian 5-HT_2 agonist, α-methyl 5-HT, resulted in an almost immediate stimulation of urea excretion, suggesting the involvement of one of the three 5-HT_2 receptor subtypes in the activation or membrane insertion of tUT. The increase in urea excretion elicited by α-methyl 5-HT was inhibited in a dose-dependent fashion by the 5-HT_2 receptor antagonist, ketanserin, which has the highest affinity for the 5-HT_{2A} receptor, with an $IC_{50} = 9.5 \times 10^{-9}$ M (McDonald and Walsh, 2004). Based on the reported differential sensitivities of the mammalian 5-HT_{2A} receptor for α-methyl 5-HT and ketanserin, it was suggested at that time that a 5-HT_{2A}-like receptor was involved in the activation of urea excretion in toadfish (McDonald and Walsh, 2004).

The primary goal of the following study was to characterize the toadfish 5-HT_{2A} receptor at a molecular level and compare its sequence similarity to other 5-HT_{2A} receptors. To gain further insight on the control of pulsatile urea excretion in toadfish, the second objective was to determine the tissues in which this receptor is predominantly expressed. The final goal of the study was to further investigate the pharmacological specificity of toadfish pulsatile urea excretion by examining the effect of ketanserin treatment, the 5-HT_{2A} antagonist used in conjunction with α-methyl 5-HT in a previous study (McDonald and Walsh, 2004), on resting rates of pulsatile urea excretion. We hypothesized that the toadfish 5-HT_{2A} receptor would show molecular homology to mammalian 5-HT_{2A} receptors that would support pharmacological conservation across different species. In addition, if the hypothesis arising from prior research that the 5-HT_{2A} receptor is involved in the regulation of toadfish pulsatile urea excretion is correct, we believe that treatment with ketanserin would inhibit natural urea pulses, and be a further test of the hypothesis.

2. Materials and methods

2.1. Experimental animals

Gulf toadfish (*O. beta*) were captured by roller trawl used by commercial shrimpers in Biscayne Bay, Florida. The toadfish were then immediately transferred to the laboratory where they were held for up to one month in 50-liter glass aquaria with flowing, aerated seawater at a temperature of 20–24 °C and a density of 10 g fish L^{-1}. Fish were treated with a dose of malachite green (final concentration 0.05 mg L^{-1}) in formalin (15 mg L^{-1}) (AquaVet) on the day of transfer to the laboratory to prevent infection by the ciliate, *Cryptocaryon irritans* (Stoskopf, 1993). Fish were fed weekly with thawed squid.

2.2. Experimental protocols

2.2.1. Tissue dissections

Tissues were excised from toadfish that had been held for one week in uncrowded conditions in outdoor 6000 L tanks seeded with the seagrass, *Thalassia testudinum* which emulates the natural environment of Gulf toadfish (Serafy et al., 1997). Toadfish were over-anesthetized with MS222 (3 g·L^{-1}) and tissues (whole brain, swim bladder (without the muscle but likely including gas gland and rete mirabile), gonad, rectum, posterior intestine, heart, muscle, liver, anterior intestine, kidney, stomach, esophagus, gill) were collected terminally. A mixture of non-reproductively active females and males were used. Different regions of the brain as defined by Medeiros et al. (2010) were also isolated (telencephalon, cerebellum, hindbrain, midbrain/diencephalon, olfactory bulb and pituitary). All tissues were frozen immediately in liquid N_2 and stored at −80 °C.

2.2.2. PCR and 5′ and 3′ rapid amplification of cDNA ends (RACE)

Total RNA was isolated from tissues following the protocol provided with the Trizol reagent (Invitrogen). Total RNA was subsequently treated with DNAse I to remove potential residual genomic DNA according to the protocol provided with the TurboDNA-free kit (Ambion). Toadfish poly(A) RNA was extracted from the total RNA using the PolyATract mRNA Isolation System III (Promega) for use in RACE reactions. cDNA was synthesized with Oligo(dT) primers from 1 μg of DNase I-treated total RNA according to the protocol provided with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). An initial fragment of 597 bp was cloned from toadfish brain cDNA using degenerate primers (Table 1) designed using alignments of zebrafish and other organisms. Reactions were performed using GoTaq DNA polymerase (Promega) and the following cycling conditions: 94 °C for 30 s, a temperature gradient of 50–70 °C for 1 min, and 72 °C for 1 min for 40 cycles. To obtain the 5′ and 3′ ends, 1 μg of poly(A) RNA was amplified into RACE-ready cDNA using the BD SMART RACE kit (Clontech). Primers for use in RACE were designed from the previously acquired toadfish sequence (Table 1). Touchdown PCR was performed using the following cycling parameters: 5 cycles of 94 °C for 30 s and 72 °C for 4 min, followed by five and then 25 additional cycles as outlined above with annealing temperatures of 70 °C and 68 °C, respectively. A second round of amplification was performed with diluted aliquots (1:100) of the initial PCR reactions using nested primers (Table 1) by repeating the cycle conditions above, except with only a 2.5 min elongation period. Products were gel-purified, cloned using the TOPO TA vector (Invitrogen) and sequenced (Geneway Sequencing; Hayward, CA. USA).

2.2.3. Quantitative real-time PCR (qPCR)

cDNA was synthesized with random hexamer primers from 1 μg of DNase I-treated total RNA from each tissue and from different segments of the brain according to the protocol provided with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) as described above. A tenfold dilution of cDNA was made up using molecular biology grade water (Sigma-Aldrich). Gene-specific primers for the toadfish 5-HT_{2A} receptor were based on sequence that was not similar to other 5-HT receptors (Table 1) and the 100 bp gene product obtained with these primers was cloned and sequence verified (Geneway Sequencing). qPCR was performed using an Mx3005P Multiple Quantitative PCR system (Stratagene) with SYBR Green qPCR Master Mix (Applied Biosystems) as the reporter dye. Cycling parameters were as follows: 95 °C for 10 min, followed by 40–50 cycles at 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. Fold-changes between tissues and brain regions were determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The standard curve of the gene of interest gave a PCR efficiency

Table 1
Primers used for cloning and qPCR of the toadfish 5-HT_{2A} receptor.

Primer	Sequence (5′ to 3′)
5-HT_{2A}-F	CTCCACSGCCTCCATCATGC
5-HT_{2A}-R	GGGCACCACATSACCAC
5-HT_{2A}-5′RACE	CAGCAGGCAGCTCCCATCTTTGAAGACC
5-HT_{2A}-n5′RACE	CTGAGTCCCAGAACAGGAATAGGCATCG
5-HT_{2A}-3′RACE	CTGTGCCATTTCTCTGGACCGATATATCGC
5-HT_{2A}-n3′RACE	CCACCACAGCCGCTTCAACTCCCACACC
qPCR 5-HT_{2A}-F	ACGCCACAAACTACTTCCTGAT
qPCR 5-HT_{2A}-R	GCCGTAATCGTAGAGAATGGTC

Abbreviations: forward primer (F); reverse primer (R); nested primer (n).

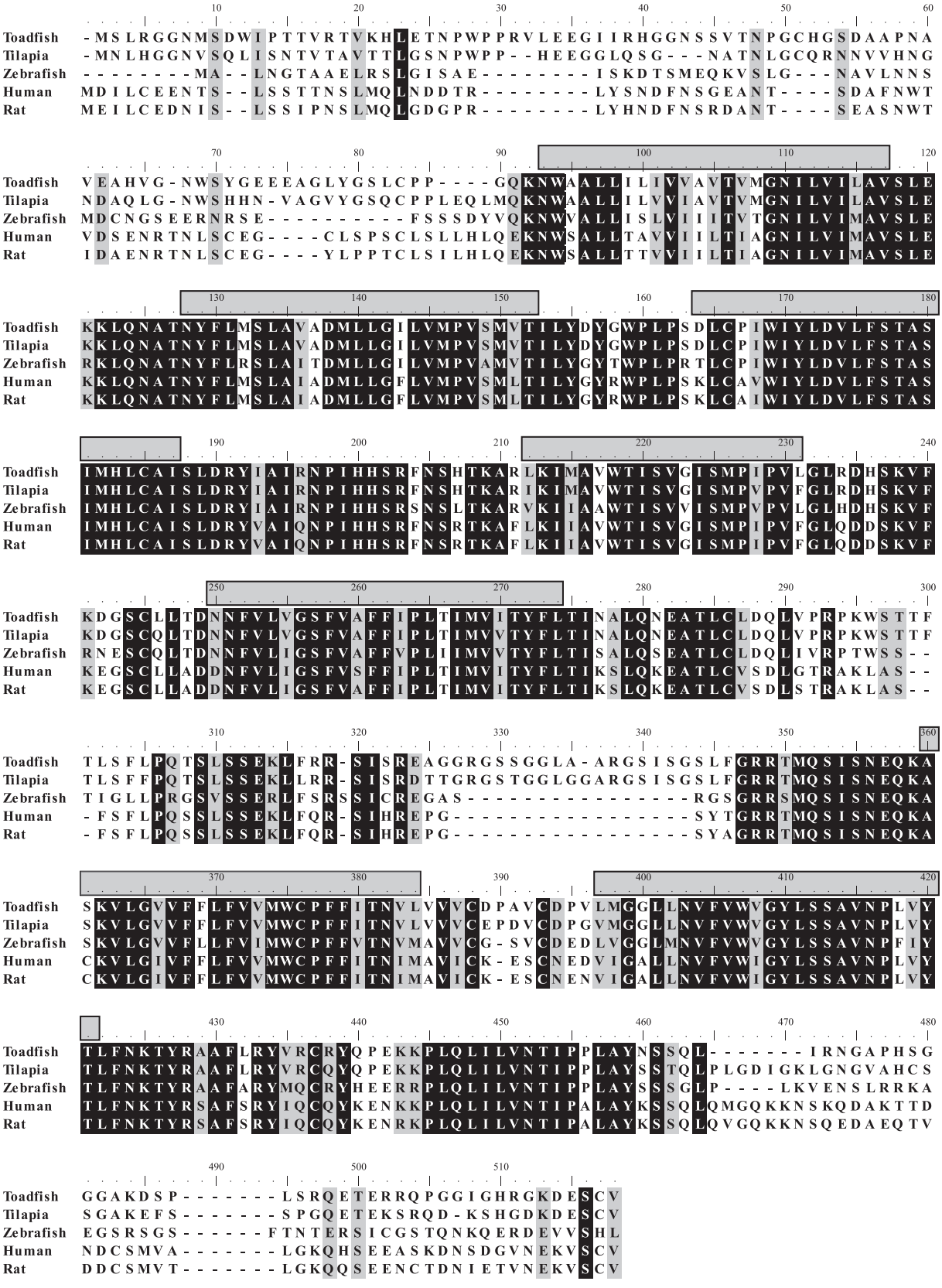

Fig. 1. Alignment of translated nucleotide toadfish 5-HT$_{2A}$ receptor protein with other 5-HT$_{2A}$ receptors: zebrafish, *Danio rerio* (XM_683178); tilapia, *Oreochromis niloticus* (XM_003449546.1); human, *Homo sapiens* (NM_000621); and rat, *Rattus norvegicus* (NM_017254.1). Black shading indicates identical amino acid residues, grey shading denotes conservative substitutions, dashes denote deletions and white areas denote non-identity. Numbers outlined in a grey box indicates a transmembrane helix, as predicted by HMMTP (v2.0; http://www.enzim.hmmtop/index.html) (Tusnády and Simon, 1998, 2001).

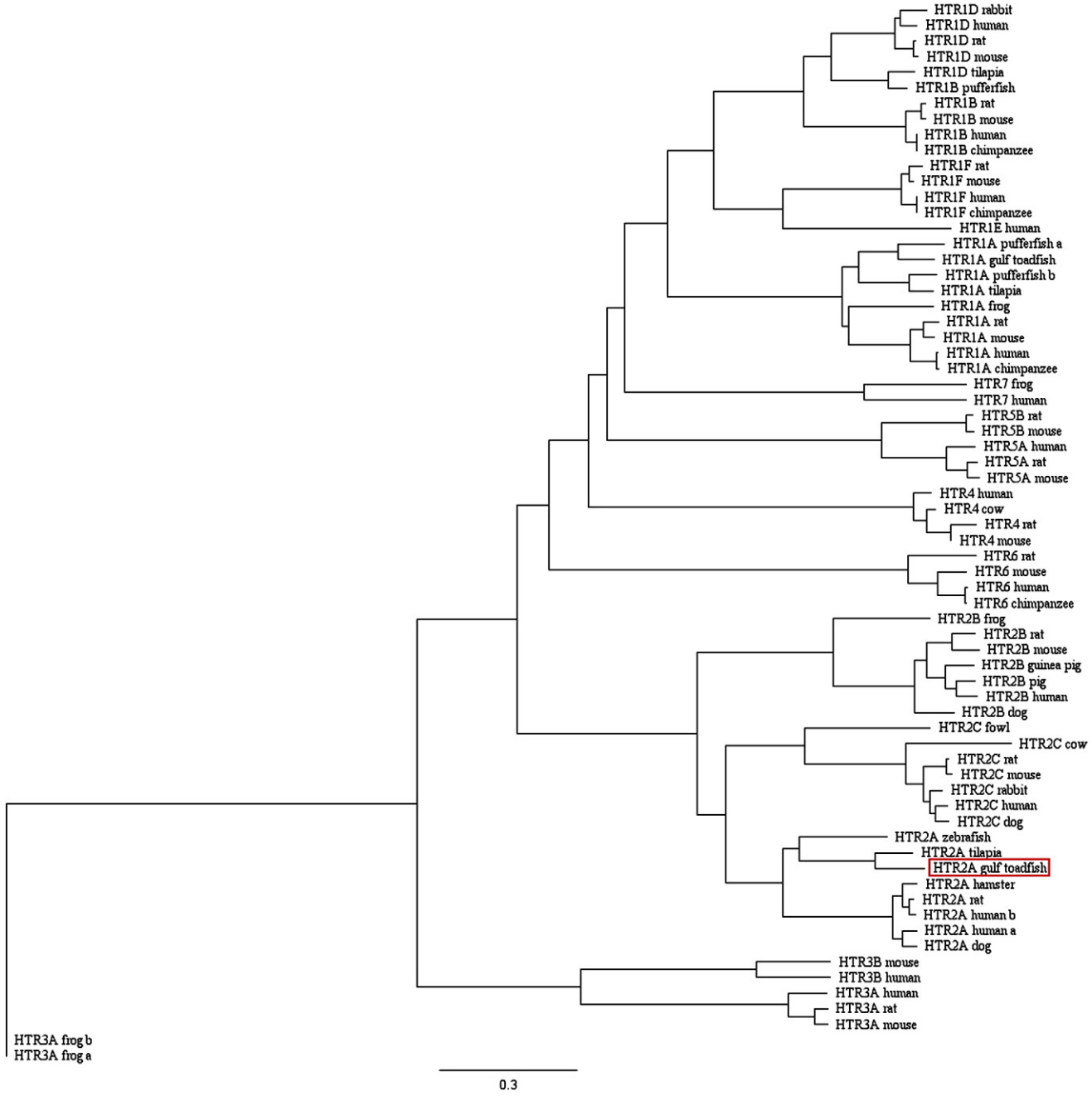

Fig. 2. Phylogenetic analysis of 65 5-HT receptor sequences which places the amino acid sequence for toadfish 5-HT_{2A} (outlined in red) in the 5-HT_{2A} receptor branch, with the highest similarity to the tilapia. Sequences were translated, aligned and a maximum likelihood tree constructed using the Jones–Taylor–Thornton substitution model and bootstrap analysis in Geneious Pro (v5.5.7).

of 106% (5-HT_{2A}; $r^2 = 0.979$). To confirm that there was no contamination or primer dimer formation contributing to the fluorescence measured, 'no reverse transcriptase' and 'no template controls' were performed. Because housekeeping gene mRNA expression varied across tissues, mRNA expression of the 5-HT_{2A} receptor was normalized to the amount of total RNA used for cDNA synthesis (1 μg). Fold-changes in mRNA expression between tissues were assessed relative to whole brain that was dissected and processed at the same time. Changes between brain regions were assessed relative to the telencephalon.

2.2.4. In vivo ketanserin treatment

Toadfish were crowded (10 fish in 10 L water) for one week to ensure the switch to ureotely (Walsh et al., 1994). After crowding, toadfish were injected intraperitoneally with either warm coconut oil alone (control 0.038 ± 0.004 (4) kg) at a dose of 5 μL coconut oil·g $fish^{-1}$ or with ketanserin (0.032 ± 0.004 (5) kg) mixed with coconut oil at a dose of 0.1 μmol·5 μL coconut oil^{-1}·g $fish^{-1}$; the dose of ketanserin was based on preliminary range-finding experiments and on the dose used in a previous study (McDonald and Walsh, 2004). Immediately post-injection, fish were left on ice as described by Morando et al. (2009) so that the coconut oil could solidify. The solidification of coconut oil will mediate the slow release of ketanserin into the fish (Morando et al., 2009). The fish were then placed in individual boxes and allowed to recover overnight after which time water flow to the boxes was stopped and water samples from boxes were collected on an hourly basis for the next 24 h using automated fraction collectors

(Bio-Rad) hooked up to a Beckman 8-channel peristaltic pump. Collected water samples were frozen at −20 °C until they were ready to be analyzed for urea and ammonia concentration. In a separate group, toadfish were treated with either coconut oil alone (control, $N=5$) or ketanserin mixed with coconut oil ($N=5$), left in boxes to recover overnight and held for the next 24 h as described above. Fish were removed from their chambers and blood samples were drawn immediately *via* caudal puncture using a 23 G needle attached to a disposable syringe rinsed with heparinized saline (50 IU mL saline^{-1}; Sigma-Aldrich) as described by Medeiros et al. (2010). Each fish was sampled within a min period so that plasma cortisol levels were not the result of sampling stress. Collected blood samples were centrifuged at 16,000 *g* for 2 min, the plasma decanted, frozen immediately in liquid nitrogen (N_2), and then stored at −80 °C until measured for cortisol concentration.

2.3. Analytical techniques

Water urea and ammonia concentrations were determined using the methods outlined by Rahmatullah and Boyde (1980) and Ivancic and Degobbis (1984), respectively. Plasma cortisol concentrations were measured using a cortisol radioimmunoassay kit from MP Biomedicals with cortisol standards diluted by half to approximate the appropriate plasma protein range for fish.

2.4. Statistical analyses

Analyses were conducted using SigmaStat and/or SigmaPlot (SPSS, Inc.). Data are given as means ± 1 S.E.M. (N), where N = the number of fish. In the case of the mRNA expression data, comparisons were conducted using a one-way ANOVA in conjunction with a Holm–Sidak or Fisher LSD multiple comparison test ($P<0.05$). If the data were not normally distributed even after log transformation, an ANOVA based on ranks was conducted followed by a Dunn's multiple comparison test ($P<0.05$). Differences between two means were measured using a Student's unpaired two-tailed *t*-test ($P<0.05$).

3. Results

The full-length toadfish 5-HT$_{2A}$ receptor (GenBank Accession # FJ611960.2) is a 1490 bp transcript that encodes a 496 amino acid sequence. It shares 80% and 57% sequence identity to the tilapia and zebrafish 5-HT$_{2A}$ receptors, respectively, and 58% identity to mammalian 5-HT$_{2A}$ receptors (Fig. 1). Predicted protein folding using the HMMTP prediction system revealed 7 transmembrane regions with the N-terminus on the extracellular side (Fig. 1). Interestingly, there is a 15 amino acid insertion (amino acids 328–343), predicted to be located intracellularly, within the toadfish and tilapia sequence that is not found within the zebrafish or the mammalian sequences (Fig. 1). Phylogenetic analysis of all 5-HT receptors resulted in placing the amino acid sequence for toadfish 5-HT$_{2A}$ in the 5-HT$_{2A}$ receptor clade (Fig. 2). The toadfish sequence had the highest similarity to the tilapia. Of note, there were two sub-branches in the 5-HT$_{2A}$ grouping, with the teleosts and terrestrial vertebrates separated.

Toadfish 5-HT$_{2A}$ receptor mRNA expression was found to be highest in the swim bladder, in which transcript levels were approximately 100-fold greater than those measured in whole brain (Fig. 3A). Expression was second highest in the gonad, which had approximately 30-fold greater transcript than whole brain. All other tissues tested had mRNA expression levels that were significantly less than whole brain, with the latter parts of the intestine (posterior intestine and rectum), the heart, muscle and gill expressing toadfish 5-HT$_{2A}$ receptor at levels that were 10–30% of that present in whole brain (Fig. 3B, C). The liver, kidney and the early segments of the gastrointestinal tract, *i.e.*, the stomach, esophagus and anterior intestine, had transcript levels that were only 4–7% of whole brain (Fig. 3C). Toadfish 5-HT$_{2A}$ receptor

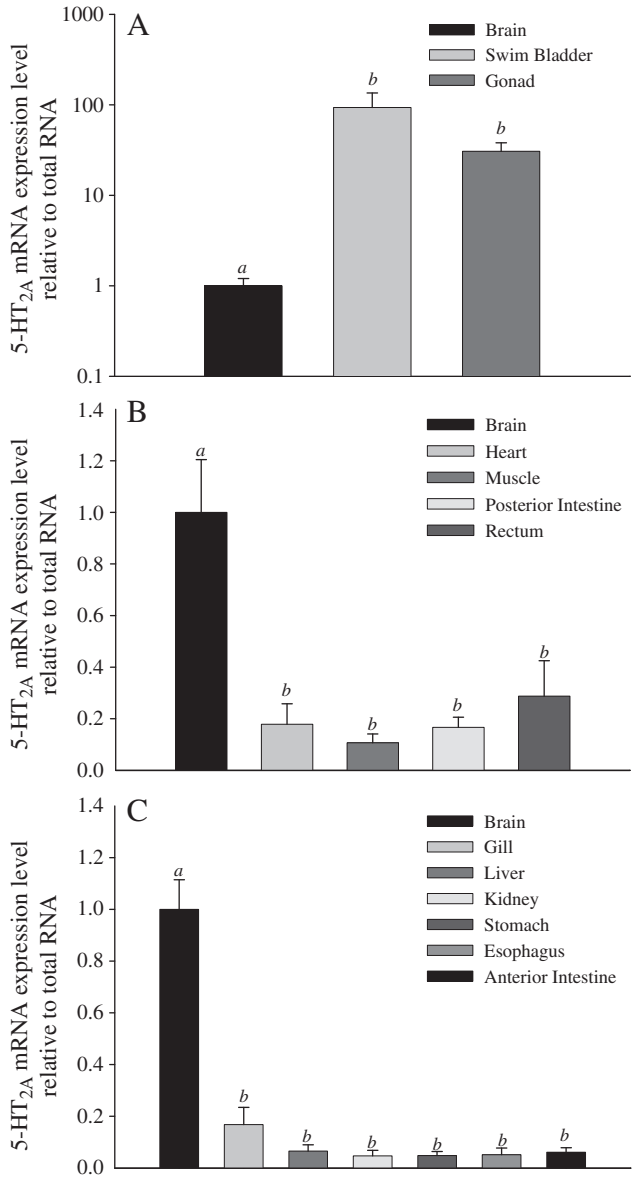

Fig. 3. (A) The mRNA expression of the 5-HT$_{2A}$ receptor is highest in the swim bladder and gonad, followed by the brain. (B) 5-HT$_{2A}$ receptor expression is significantly lower in rectum, posterior intestine, heart and muscle compared to the brain. (C) 5-HT$_{2A}$ receptor expression is significantly higher in brain that in liver, anterior intestine, kidney, stomach, esophagus and gill. Values are means ± S.E.M. ($N=5$–6); different letter denotes significant difference compared to brain; $P<0.05$.

mRNA expression within the brain tended to be highest in the hindbrain region, followed by the telencephalon and midbrain/diencephalon (Fig. 4). All three regions expressed significantly more transcript than either the olfactory bulb or pituitary, in which transcript levels of 5-HT$_{2A}$ were not significantly detectable (Fig. 4).

Treatment with the 5-HT$_{2A}$ receptor antagonist, ketanserin, resulted in a significant decrease in the total amount of urea excreted as well as the pulsatile component of urea excretion compared to control treated fish (Fig. 5A). There was no significant change in the non-pulsatile component, which was undetectable in ketanserin-treated fish. This translated to a significantly lower pulse size (Fig. 5B) with no significant change in the pulse frequency (Fig. 5C). Plasma cortisol levels between the two groups of fish were not significantly different (Fig. 5D). Ketanserin-treated toadfish also showed a decrease in their % ureotely, consistent with a reduction in urea excretion rate with no significant change in the rate of ammonia excretion (Table 2).

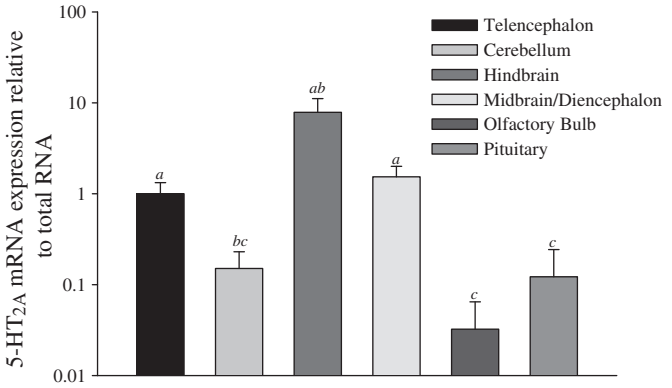

Fig. 4. The mRNA expression of the 5-HT_{2A} receptor is highest in the hindbrain, telencephalon and midbrain/diencephalon regions. The lowest levels are found in the olfactory bulb and pituitary. Brain regions are as described in Medeiros et al. (2010). Values are means ± S.E.M. ($N = 5–8$); different letter denotes significant difference compared to the telencephalon; $P<0.05$.

4. Discussion

As hypothesized, the Gulf toadfish, *O. beta*, does indeed possess a 5-HT_{2A} receptor that is similar to zebrafish and mammalian 5-HT_{2A} receptors at the amino acid level and it groups with the 5-HT_{2A} receptor subtype upon phylogenetic analysis. Whether this similarity translates to pharmacological conservation is best addressed using a preparation in which the specificity of an isolated toadfish 5-HT_{2A} receptor to mammalian pharmacological compounds could be directly tested (such as *Xenopus laevis* oocyte or human cell line expression systems). In the context of previous work on pulsatile urea excretion, this would be testing the binding kinetics of α-methyl 5-HT, the mammalian 5-HT_2 receptor agonist, or ketanserin, a mammalian 5-competitive antagonist that is most specific for the 5-HT_{2A} receptor (McDonald and Walsh, 2004). While this work has not yet been completed, Shapiro et al. (2000) have outlined that the most important residues within the human 5-HT_{2A} receptor sequence for ligand binding, due to their stabilizing interactions with the ligand, include human D155 that corresponds with toadfish D173in helix 3; human S159 that corresponds to the toadfish S177 in helix 3; human S207 and S239 that correspond to toadfish S225 in helix 4 and toadfish S257 in helix 5; human S372 and S373 that correspond to toadfish S412 and S413 in helix 7; and human F240, F340, W336, and W367 that correspond to toadfish F258, F379, W375, and W407. Among these very important residues, there is 100% conservation in Gulf toadfish, suggesting that, in addition to molecular conservation, there would likely be pharmacological conservation.

The toadfish 5-HT_{2A} mRNA expression pattern is consistent with the functions outlined for this receptor in mammals and other teleost fish. Specifically, the 5-HT_{2A} receptor has been proposed to play a role in the regulation of intestinal contraction in teleosts (Burka et al., 1989); therefore, the higher expression patterns measured in parts of the toadfish intestine (*i.e.*, posterior intestine, rectum) could reflect this capacity in toadfish. The 5-HT_{2A} receptor has also been shown to play a role in vascular smooth muscle contraction and cardiovascular control in both mammals and the Gulf toadfish (Nagatomo et al., 2004; Brattelid et al., 2007; McDonald et al., 2010); functions that could be reflected in the presence of 5-HT_{2A} transcript in the toadfish heart. A 5-HT_{2A} receptor is believed to be responsible in mediating stimulation of gonadotropin-releasing hormone and/or gonadotropin hormone release in teleost fish (Somoza et al., 1988; Somoza and Peter, 1991; Khan and Thomas, 1992; Senthikumaran et al., 2001), two hormones that are involved in mediating sex hormone release from gonad. To our knowledge, the role of the 5-HT_{2A} receptor at the level of the teleost gonad has not been investigated, but may not be surprising given its functional predominance within the hypothalamic–pituitary–gonadal axis of other fish. In mammalian brain, the highest density of 5-HT_{2A} receptor, using autoradiography with [^{3}H]-M100907, a selective 5-HT_{2A} antagonist, has been shown to be throughout neocortical regions with low levels in the hippocampus, basal ganglia and thalamus and undetectable levels in the cerebellum and brainstem (Hall et al., 2000). In contrast to human brainstem, the mRNA expression of the 5-HT_{2A} receptor was elevated in the toadfish hindbrain. However, consistent with the density of 5-HT_{2A} in the human neocortex and cerebellum,

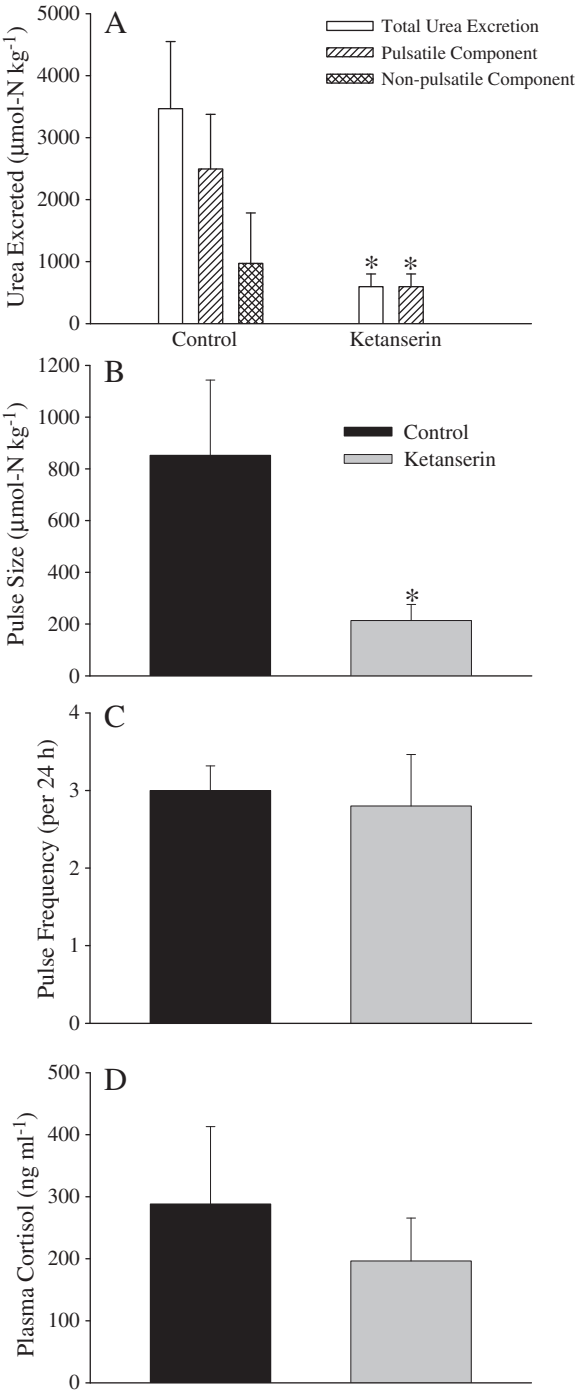

Fig. 5. (A) Ketanserin treatment resulted in a significant reduction in total urea excretion and the pulsatile component of urea excretion that was reflected as a significant reduction in (B) pulse size but not (C) pulse frequency. (D) There was no significant difference in plasma cortisol levels between the two treatment groups. Values are means ± S.E.M. ($N = 7$ control, $N = 8$ ketanserin-treated); $^*P<0.05$ compared to control.

Table 2
Rates of nitrogen waste excretion and % ureotely in control and ketanserin-treated fish.

	Control	Ketanserin
Urea excretion rate ($\mu mol\text{-}N \cdot kg^{-1} \cdot h^{-1}$)	144.4 ± 45.1	24.9 ± 8.5*
Ammonia excretion rate ($\mu mol\text{-}N \cdot kg^{-1} \cdot h^{-1}$)	37.7 ± 18.5	55.8 ± 31.2
% Ureotely	84.1 ± 4.9	44.8 ± 12.1*

Values are means ± 1 S.E.M.
* $P<0.05$ compared to respective control value.

toadfish 5-HT_{2A} mRNA expression levels are relatively high in the telencephalon and midbrain/diencephalon regions and low in the cerebellum.

Interestingly, the area highest in toadfish 5-HT_{2A} receptor mRNA expression was not the brain but the swim bladder. The swim bladder was stripped of its skeletal muscle, so the 5-HT_{2A} receptor that has been shown to be present in rat skeletal muscle (Guillet-Deniau et al., 1997; Hajduch et al., 1999) should be of no consequence. The gas gland and associated rete mirabile were likely included in the swim bladder preparation (Fänge and Wittenberg, 1958), and so elevated toadfish 5-HT_{2A} receptor transcript may be present in that capacity as rete mirabile in both mammals and fish have been shown to respond to 5-HT (Myhre et al., 1976; Diéguez et al., 1987). However, we believe that the exceedingly high levels of transcript in the swim bladder, in addition to elevations in gonad and the hindbrain, may suggest a larger role for 5-HT_{2A} in the toadfish, as all three of these regions are in fact interrelated in this fish. Specifically, the swim bladder of fish from the family Batrachoididae (toadfish and midshipman) acts as a sonic apparatus, allowing the fish to produce sound, both to communicate aggression (grunts) as well as reproductive availability and prowess (boat whistles) (reviewed by Bass and McKibben, 2003). Furthermore, this sound production is believed to be controlled by the vocal motor neurons of the hindbrain (Bass et al., 2008) and is intimately associated with reproductive status and levels of reproductive hormone (Modesto and Canário, 2003; Remage-Healey and Bass, 2004; Lee and Bass, 2005). Thus, we suggest that the elevated 5-HT_{2A} receptor transcript levels in the swim bladder, hindbrain and gonad could suggest a potential functional connection in terms of the role of 5-HT in the regulation of sound production.

Toadfish 5-HT_{2A} receptor transcript was present in the gill, albeit at only 16% the levels measured in the brain. The adaptive advantage of toadfish pulsatile urea excretion is currently unknown; however, it has been suggested that urea production and pulsatile urea excretion could play a role in a predator avoidance strategy that may be particularly important in a sound-producing fish such as the toadfish. In brief, a study by Barimo and Walsh (2006) showed that ammonia, the typical nitrogen waste of teleost fish, is an important chemical attractant for predators such as the grey snapper (*Lutjanus griseus*), one of the top predators of Gulf toadfish. Barimo and Walsh (2006) went on to show that the grey snapper responds more aggressively to a plume of ammonia than it does to a plume containing a mixture of ammonia and urea, or a plume of urea alone, suggesting that the excretion of urea may somehow mask the chemical signal elicited by ammonia alone. Work done by Sloman et al. (2005) showed a potential role of pulsatile urea excretion in silent, chemical communication when two toadfish, allowed to interact, pulsed urea within 1 h of each other (compared to random urea excretion when toadfish were held apart). Interestingly, the study also showed that urea itself was not the signaling molecule, suggesting that whatever the toadfish were responding to behaviorally was released at the same time as the urea pulse (Sloman et al., 2005). Perhaps 5-HT_{2A} receptor transcript in the swim bladder, gonad, hindbrain and gill reflects a link between sound production, which makes a fish more susceptible to detection by predators, and an adaptive predator avoidance strategy by chemical crypsis and silent communication through pulsatile urea excretion. Incidentally, sound production by the swim bladder has been shown to be inhibited in response to predators in Gulf toadfish (Remage-Healey et al., 2006).

The involvement of the toadfish 5-HT_{2A} receptor in the control of pulsatile urea excretion was first proposed because arterial injections of the mammalian 5-HT_2 agonist, α-methyl 5-HT, resulted in a stimulation of urea excretion that was significantly inhibited by pre-treatment with the 5-HT_{2A} receptor antagonist, ketanserin, even at ketanserin doses that were 2-orders of magnitude less than α-methyl 5-HT (McDonald and Walsh, 2004). Both the IC_{50} for ketanserin established in that study (9.5×10^{-9} M) and the reported differential binding affinities of the mammalian 5-HT_{2A} receptor for α-methyl 5-HT and ketanserin (Barnes and Sharp, 1999), suggested the involvement of the 5-HT_{2A} receptor over other 5-HT_2 receptor subtypes (McDonald and Walsh, 2004). The inhibitory effect of ketanserin on urea excretion is robust; in addition to toadfish, treatment with ketanserin has also been shown to inhibit urea excretion in the mangrove rivulus (Rodela and Wright, 2006). Furthermore, in toadfish of the present study, ketanserin treatment inhibited spontaneously occurring urea pulses (*i.e.*, pulses that were not pharmacologically induced by α-methyl 5-HT as in McDonald and Walsh, 2004), resulting in a significant inhibition in the pulsatile component of urea excretion and a decrease in urea pulse size with no change in frequency. Interestingly, an inhibition in urea pulse size without a change in urea pulse frequency has also been found in toadfish with elevated plasma cortisol levels; a finding that is hypothesized to be due to cortisol's interference with 5-HT receptor-mediated activation of tUT (McDonald et al., 2004). Plasma cortisol concentrations of fish of the present study were not significantly different, and so the inhibition in pulsatile urea excretion measured in these fish is due to the inhibitory effect of ketanserin alone.

The present study demonstrated that, indeed, the toadfish does have a 5-HT_{2A} receptor that is similar to mammalian receptors on a molecular and likely on a pharmacological basis, that the toadfish 5-HT_{2A} receptor is expressed at high levels in swim bladder, gonad and hindbrain in addition to the gill and that the inhibitory effect of ketanserin, a 5-HT_2 receptor antagonist most selective for the 5-HT_{2A} subtype is robust. Still, work is clearly needed to establish the specificity of α-methyl 5-HT and ketanserin for the toadfish 5-HT_{2A} receptor so that this receptor subtype can be established as mediating the regulation of toadfish pulsatile urea excretion. Furthermore, it would be interesting to determine whether 5-HT_{2A} mRNA expression levels in swim bladder, gonad, hindbrain and gill in response to social or predation stressors correlate with tUT mRNA expression, urea production and rates of pulsatile urea excretion.

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