

The serotonin subtype 1A receptor regulates cortisol secretion in the Gulf toadfish, *Opsanus beta*

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ABSTRACT

It is well established that serotonin (5-HT; 5-hydroxytryptamine) plays a role in mammalian regulation of the hypothalamic–pituitary–adrenal (HPA) axis via the 5-HT receptor subtype 1A (5-HT_{1A}). To date, there has not been a comprehensive investigation of the molecular, pharmacological and physiological aspects of the 5-HT_{1A} receptor and its role in the activation of the hypothalamic–pituitary–interrenal (HPI) axis in teleost fish. The 5-HT_{1A} receptor of the Gulf toadfish (*Opsanus beta*) was cloned and sequenced, showing 67.5% amino acid similarity to the human homologue. The 5-HT_{1A} receptor was distributed throughout the brain, with the whole brain containing significantly higher levels of 5-HT_{1A} mRNA compared to all other tissues and the midbrain/diencephalon region containing significantly higher levels of transcript than any other brain region. Substantial levels of transcript were also found in the pituitary, while very low levels were in the kidney that contains the interrenal cells. *Xenopus* oocytes injected with toadfish 5-HT_{1A} receptor cRNA displayed significantly higher binding of [³H]5-HT that was abolished by the mammalian 5-HT_{1A} receptor agonist, 8-OH-DPAT, indicating a conserved binding site of the toadfish 5-HT_{1A} receptor and a high specificity for the agonist. Supporting this, binding of [³H]5-HT was not affected by the mammalian 5-HT_{1B} receptor agonist, 5-nonyloxytryptamine, the 5-HT₇ receptor antagonist, SB269970, or the 5-HT₂ receptor agonist, α -methylserotonin. Confirming these molecular and pharmacological findings, intravenous injection of 8-OH-DPAT stimulated the HPI axis to cause a 2-fold increase in circulating levels of cortisol. The present study of the 5-HT_{1A} receptor in a single teleost species illustrates the high conservation of this 5-HT receptor amongst vertebrates.

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1. Introduction

The monoamine neurotransmitter, serotonin (5-HT; 5-hydroxytryptamine), affects many systems in the body, mediating the physiological processes that regulate anger, mood, sleep, appetite, and even learning (Fuller, 1990; Uphouse, 1997; Barnes and Sharp, 1999). Serotonergic activation of the hypothalamic–pituitary–adrenal (HPA) axis, an important component of the mammalian stress response, results in an increase in levels of corticotropin-releasing hormone (CRH) from the hypothalamus and stimulates the secretion of adrenocorticotropic-releasing hormone (ACTH) from the pituitary, which then activates the secretion of glucocorticoids, such as cortisol, from the adrenal gland (Calogero et al., 1990). In addition to the indirect stimulation of glucocorticoid secretion via CRH and ACTH (Calogero et al., 1990), 5-HT also activates the release of cortisol directly by acting on the adrenal gland; however, this increase is thought to be independent of the HPA

axis with 5-HT acting as a local paracrine factor (Alper, 1990). Reciprocally, it has been shown that brain 5-HT synthesis and turnover is greatly impacted by stressful events (Bliss et al., 1972), which, in turn, will feedback on the HPA axis. Thus, brain 5-HT plays an integral role in a complex neuroendocrine loop serving to maintain homeostasis and promote acclimation during physiological and/or environmental challenges. It was not until the last few decades that the connection between the HPA axis, 5-HT and the 5-HT receptor subtype 1A (5-HT_{1A}) was made (Lorens and Van de Kar, 1987; Fuller, 1992).

Within the CNS, 5-HT binds to several different types of 5-HT receptors (HTRs), located both pre- and postsynaptically. To date, seven families of HTRs have been identified (5-HT₁–5-HT₇), with a total of 14 subtypes having been characterized (Hoyer et al., 2002). All families are G protein-coupled receptors, with the exception of 5-HT₃, which is a ligand-gated ion channel. The 5-HT₁ family inhibits the formation of cyclic AMP (cAMP), whereas 5-HT_{4,6,7} families stimulate the production of cAMP. The 5-HT₂ family communicates via the second messenger phospholipase C, while the mechanism whereby the 5-HT₅ family acts remains a little obscure (see Hoyer et al., 2002). While all of these receptors have a specific and important purpose, the 5-HT_{1A} receptor is one of the most

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abundant subtypes of 5-HT receptors in the mammalian brain (Albert et al., 1990) and, being the first HTR cloned (Kobilka et al., 1987), has been the most studied. The G-protein-coupled receptor employs a G_i/G_o transduction system that primarily decreases adenylate cyclase formation and/or increases K^+ conductance (Barnes and Sharp, 1999). These mechanisms are postulated to inhibit firing of the postsynaptic cell, and it has been observed that 5-HT, mediated by the 5-HT_{1A} receptor, exerts a predominantly inhibitory effect on neuron firing rate in many areas of the brain (Clark et al., 1987; Araneda and Andrade, 1991; Kow et al., 1992). There are two populations of 5-HT_{1A} receptors: somatodendritic autoreceptors located mainly in the rostral raphe nucleus region of the medulla and postsynaptic receptors, located in the projection areas of the raphe nuclei, such as the telencephalon and hypothalamic/pituitary region. The somatodendritic 5-HT_{1A} autoreceptors regulate the release of 5-HT by the presynaptic neuron, ultimately affecting the stimulation of 5-HT_{1A} receptors on the postsynaptic cell and thus the activation of the 5-HT_{1A} neuron projection areas in the brain (Lanfumeu and Hamon, 2000; Albert and Lemonde, 2004). Agonists of mammalian 5-HT_{1A} receptors, such as 8-hydroxy-2-(di-n-propylamino) tetraline (8-OH-DPAT), have been found to elevate plasma corticosteroid levels in mammals (Dinan, 1996). Depending on the location of receptor, this endpoint can be achieved in several different ways, i.e., when applied directly to the rat hypothalamus, 8-OH-DPAT stimulates the release of CRH, though when applied directly to the pituitary, ACTH is released (Calogero et al., 1990). There is also evidence for the presence of a negative feedback loop whereby increased circulating levels of cortisol inhibit 5-HT_{1A} receptor activity (Zhong and Ciarello, 1995) in addition to directly inhibiting the release of ACTH and CRH (Canny et al., 1989; Delbende et al., 1992).

Work done on teleost fish investigating the interaction between 5-HT and the teleost homologue of the HPA axis, the hypothalamic–pituitary–interrenal (HPI) axis, has mostly focused on how these systems work together during social interactions (Winberg and Nilsson, 1993; Larson et al., 2003; Clotfelter et al., 2007). Social stressors, such as subordination, elevate brain 5-HT activity as indicated by brain 5-hydroxyindoleacetic acid (5-HIAA; the major 5-HT metabolite) concentrations and 5-HIAA:5-HT ratios (Winberg and Nilsson, 1993). At the same time, socially subordinate fish display elevated plasma cortisol levels (Ejike and Schreck, 1980) and increased interrenal cell size, suggesting a chronic activation of the HPI axis (Noakes and Leatherland, 1977). Work done on the brain of Arctic Charr (*Salvelinus alpinus*) suggested that, like in mammals, there are a multitude of 5-HT receptors in teleost fish to carry out the actions of 5-HT (Winberg and Nilsson, 1996). Furthermore, similar to its action in mammals, the 5-HT_{1A} receptor agonist, 8-OH-DPAT, has been shown to stimulate the release of cortisol in the rainbow trout (*Oncorhynchus mykiss*, Winberg et al., 1997), with a binding affinity (K_i) similar to what is measured in mammals (Zifa and Fillion, 1992).

While molecular evidence for the presence of 5-HT_{1A} receptors in fish (Yamaguchi and Brenner, 1997; Wang and Tsai, 2006; Airhart et al., 2007) exist and studies have provided pharmacological evidence for a role for 5-HT_{1A} in teleosts (Winberg et al., 1997; Clotfelter et al., 2007; Smith and Combs, 2008) no single study has reported molecular, pharmacological and physiological characterization of the teleost 5-HT_{1A} receptor. Thus, previous to this study there has not been an extensive investigation that has determined conclusively that the 5-HT_{1A} receptor in fish is pharmacologically similar to that found in mammals and that it plays a role in the activation of the HPI axis. Furthermore, the distribution of 5-HT_{1A} transcript within the HPI axis has not been investigated in teleost fish, although it can be hypothesized based on mammalian studies that most of the 5-HT_{1A} transcript would be found in the pituitary gland (Chalmers and Watson, 1991; Lopez et al., 1998; Drevets et al.,

2007; Kumar and Mann, 2007). Preliminary pharmacological evidence on the Gulf toadfish, *Opsanus beta*, a benthic, marine teleost fish suggested that a 5-HT_{1A}-like receptor may not be involved in the regulation of the HPI axis, as fish injected with 8-OH-DPAT failed to show an increase in plasma cortisol levels that exceeded the increase measured in saline-injected controls (McDonald and Walsh, 2004). However, it was speculated in that study that the high circulating cortisol levels experienced typically by cannulated toadfish ($200\text{--}400 \times 10^{-9} \text{ g ml}^{-1}$) may have reduced the sensitivity of the 5-HT_{1A} receptor, making it difficult to further stimulate the HPI axis in these fish. We hypothesize now that the 5-HT_{1A} receptor is indeed present in toadfish, but perhaps a higher dose of 8-OH-DPAT is necessary to overcome this potential desensitization. Thus, the objectives of this study were to examine the existence of the 5-HT_{1A} receptor in the Gulf toadfish, and specifically determine its distribution within the HPI axis, and at the same time provide functional evidence linking the toadfish 5-HT_{1A} receptor to the activation of the HPI axis. To do this, we first determined the full length nucleotide sequence of the toadfish 5-HT_{1A} receptor, we then evaluated the distribution of 5-HT_{1A} receptor transcript levels within different tissues, including those of the HPI axis. Lastly, we set out to provide pharmacological and functional evidence linking the 5-HT_{1A} receptor to HPI axis regulation.

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 in the summer of 2007, after which they were then immediately 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) on the day of transfer to the laboratory in order to prevent infection by the ciliate *Cryptocaryon irritans* (Stoskopf, 1993). The fish were kept in 50-l glass aquaria with flowing, aerated seawater at a temperature of 24–29 °C and were fed weekly with squid.

2.2. Experimental protocol

2.2.1. RNA extractions

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 were collected terminally, frozen immediately in liquid N_2 and stored at -80°C . 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 Turbo-DNA-free kit (Ambion).

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

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 779 bp was cloned from toadfish brain cDNA using degenerate primers (Table 1) designed using alignments of other teleosts. 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

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

Primer	Sequence (5' → 3')	Product Size (bp)
5-HT _{1A} -F ^a	TAYCARGTSYNTAAAYAACTG	857
5-HT _{1A} -R ^a	TANCCNARCCARTTRATNAC	
5-HT _{1A} -5' RACE	GTTTACGCACGGAGAGTTGGCTTTAGG	786
5-HT _{1A} -n5' RACE	GATGAACAGGTCGCAGAGCTCCTG	306
5-HT _{1A} -3' RACE	CAGGAGCTCTCGACCTGTTCATC	1100
5-HT _{1A} -n3' RACE	CTCTTGATTAGCGTGACTTGGTTAA TTGGTTTC	915
qPCR 5-HT _{1A} -F	ACGAAACCATGAACGAAAGG	119
qPCR 5-HT _{1A} -R	AAGGGAAAGCCAGCAGAAGAT	
18S-F	GCTCGTAGTTGGATCTCGG	166
18S-R	GCCTGCTTTGAACACTC	
ORF 5-HT _{1A} -F	ATGGATTTTGTGACAAGCAGCAACG	1253
ORF 5-HT _{1A} -R	TTAAGCTCTGTGGAATTTGC	

Abbreviations: forward primer (F); reverse primer (R); nested primer (n); open reading frame (ORF).

^a Primer sequences used for initial cloning of toadfish 5-HT_{1A} fragment, designed from partially conserved regions of other teleost 5-HT_{1A} aligned sequences.

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: five 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 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).

2.2.3. Quantitative PCR (qPCR)

A tenfold dilution of cDNA was made up using molecular biology grade water (Sigma-Aldrich). Housekeeping gene primer sequences for 18S were obtained from Grosell et al. (2009). qPCR was performed using a Mx4000 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 30 s, 60 °C for 30 s and 72 °C for 30 s. At least 6 separate biological replicates, representing cDNA isolated from at least 6 individual fish, were used for each sample. Fold-changes between tissues and brain regions were determined using the 2^{-ΔΔCt} method (Livak and Schmittgen, 2001). Expression of 18S appeared stable across different brain regions, but varied significantly among the other tissues examined throughout the organism. Therefore, within the brain 5-HT_{1A} expression was normalized to 18S, while expression in other tissues 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 the brain and changes between brain regions were assessed relative to the telencephalon.

2.2.4. Binding and pharmacological specificity for toadfish 5-HT_{1A} receptors expressed in *Xenopus* oocytes

The full-length toadfish 5-HT_{1A} receptor was cloned into the pGH19 vector (a kind gift from Dr. Gerhard Dahl, University of Miami, Miller School of Medicine, Miami, FL, USA) and linearized with *Xho*I. cRNA was synthesized using the mMessage mMachine T7 Transcription Kit (Ambion) and microinjected (1 µg) into collagenase-treated, defolliculated *Xenopus laevis* oocytes as described previously (Lindley et al., 2007). Parallel groups of oocytes were injected with water as a negative control. The binding experiment

was conducted 36 h after injection and involved five parts: (i) binding of 1.35 × 10⁻⁹ I⁻¹ [³H]-5-HT by 5-HT_{1A}-injected oocytes compared to water-injected controls; (ii) the displacement or competition of 1.35 × 10⁻⁹ I⁻¹ [³H]5-HT binding by 1.35 × 10⁻⁹ I⁻¹ 8-hydroxy-2-(di-n-propylamino) tetraline (8-OH-DPAT; Sigma-Aldrich), an agonist specific for mammalian 5-HT_{1A} receptors; (iii) evaluation of 5-HT binding affinity using [³H]5-HT concentrations varying from 0.0 to 2.0 × 10⁻⁹ I⁻¹; (iv) determination of an EC50 for 8-OH-DPAT using the displacement or competition of 1.00 × 10⁻⁹ I⁻¹ [³H]5-HT binding by 8-OH-DPAT ranging in concentration from 0.0 to 10.0 × 10⁻⁹ I⁻¹; and (v) the displacement or competition of 1.0 × 10⁻⁹ I⁻¹ [³H]5-HT binding by 1.80 × 10⁻¹¹ mol l⁻¹ 8-OH-DPAT, SB269970 (a 5-HT₇ antagonist; Sigma-Aldrich), α-methylserotonin (a 5-HT₂ agonist; Sigma-Aldrich), or 5-nonyloxytryptamine (a 5-HT_{1B} agonist; Tocris). A separate control treatment was evaluated with 5-nonyloxytryptamine due to it only being soluble in 100% EtOH; serial dilutions put the final EtOH concentration at 0.01%. Injected oocytes were pre-incubated in buffer (in mM: 200 mannitol, 2 KCl, 1 MgCl₂, 1 CaCl₂, 10 Hepes, 5 Tris, pH 7.4) for 15 min and then transferred to a binding solution consisting of the pre-incubation buffer containing [³H]5-HT (American Radiolabeled Chemicals, Inc.) with or without the appropriate serotonin receptor agonist or antagonist. Oocytes were incubated for 60 min and then removed from the incubation bath and rinsed twice with pre-incubation buffer. Oocytes were placed individually into 20 ml scintillation vials and immediately digested in 10% SDS (1 ml) overnight. After digestion, 10 ml of Ecolume scintillation fluor (MP Biomedical) was added to each vial and radioactivity was measured by liquid scintillation counting (Tri-Carb 2100TR Liquid Scintillation Counter, Packard). Non-specific binding was removed before making comparisons from all treatments except when comparing the binding of [³H]5-HT in water-injected oocytes to those injected with 5-HT_{1A} cRNA.

2.2.5. In vivo injection of 8-OH-DPAT to determine potential presence of the 5-HT_{1A} receptor in gulf toadfish

Toadfish (80 ± 5 g; N = 9) were anesthetized with MS222 (1 g l⁻¹; Argent Chemical Laboratories) and fitted with caudal vein catheters inserted using surgical techniques previously described (Wood et al., 1997; McDonald et al., 2000). Catheterized fish were allowed to recover for at least 40 hours before injection with 8-OH-DPAT (Sigma-Aldrich). Responsiveness of the HPI axis to two different doses of 8-OH-DPAT (3.25 and 16.25 mg kg⁻¹ ml⁻¹ saline, which represented doses calculated to achieve circulating 8-OH-DPAT concentrations that were 10- and 50-times higher than circulating levels of 5-HT, respectively) were tested. These doses were based on the lack of an effect of 8-OH-DPAT injection to stimulate a cortisol response that was significantly different than that elicited by serial blood sampling alone at a dose of 0.325 mg kg⁻¹ ml⁻¹ saline (McDonald and Walsh, 2004). Immediately following the collection of a 200 µl blood sample (t = 0 h), 8-OH-DPAT was administered via the caudal catheter. Subsequent blood samples (200 µl) were taken at t = 0.25, 0.5, 1, 2, and 4 h post-8-OH-DPAT injection. After each sample was spun down, red blood cells were resuspended in 200 µl saline (150 mmol l⁻¹ NaCl) and injected back into the fish. Collected samples were centrifuged at 10,000g for 5 min and the plasma decanted. Plasma from each sample was flash frozen in liquid N₂, and then stored at -80 °C until being used to evaluate circulating levels of the stress hormone, cortisol, and products of gluconeogenesis such as circulating levels of urea, protein and glucose.

2.2.6. Organic compound assays

Plasma glucose concentrations were measured using a commercial Hexokinase kit (Stanbio Laboratory), with standards ranging from 0 to 25 mmol l⁻¹. Circulating levels of protein were assessed

using Bradford Assay (Sigma-Aldrich), with bovine protein standard (Sigma-Aldrich) diluted to a toadfish-appropriate range of 0–0.06 g 100 ml⁻¹. Plasma urea levels were measured using the diacetyl monoxime method of Rahmatullah and Boyde (1980),

with changes to reagent strength appropriate for plasma urea concentrations found in toadfish. Plasma cortisol was quantified using a ¹²⁵I RIA kit (MP Biomedical), with the standards diluted by half to be appropriate for the plasma protein range of toadfish.

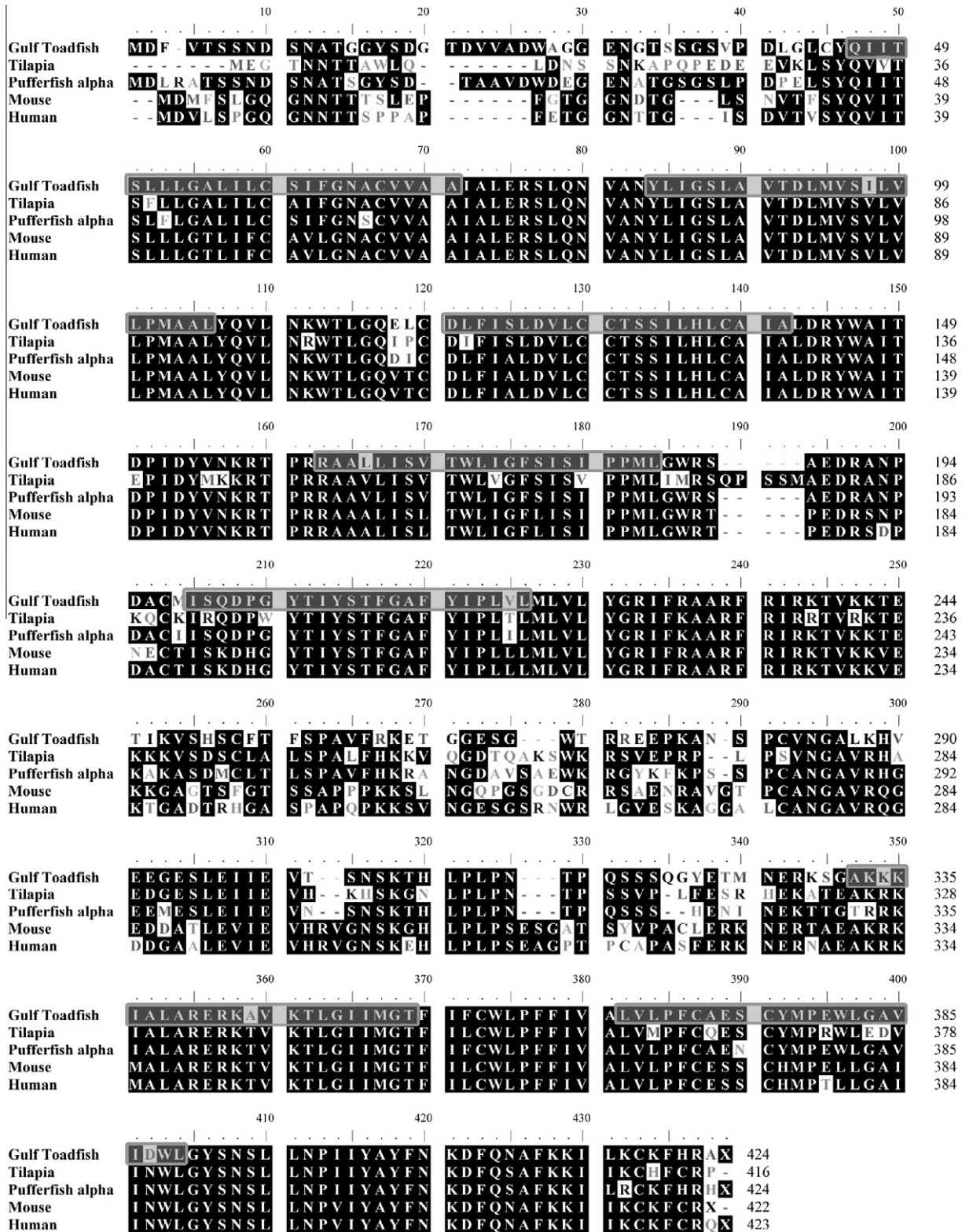


Fig. 1. CLUSTAL (v1.8) amino acid alignment of translated nucleotide 5-HT_{1A} receptor sequences from human (*homo sapiens*), NM000524; mouse (*Mus musculus*), MMU39391; Fugu (*Takifugu rubripes*), X95936; Tilapia (*Oreochromis mossambicus*), AY219038; and Gulf toadfish (*Opsanus beta*), FJ769221. Outlined amino acids indicate a transmembrane helix, as predicted by HMMTP (v2.0; <http://www.enzim.hu/hmmtop/index.html>).

2.2.7. Statistical analyses

All binding curve analyses of *in vitro* data were determined using GraphPad Prism v5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. All other analyses were conducted using SigmaStat and/or SigmaPlot (SPSS, Inc.). Data are given as means ± 1 s.e.m., and N = the number of fish or oocytes. The *in vivo* response to 8-OH DPAT was analyzed with a one-way ANOVA with time as the main factor and multiple comparisons were conducted using the Holm-Sidak method ($p < 0.05$). All other comparisons were conducted using either the Student's unpaired two-tailed *t*-test ($p < 0.05$) or, if data were not

normally distributed even after log transformation, the Mann-Whitney rank sum test ($p < 0.05$).

3. Results

The toadfish 5-HT_{1A} receptor transcript (GenBank Accession# FJ769221) is 1824 nucleotides long, and codes for 423 amino acids. Upon analysis, it was determined that the predicted amino acid sequence shares 67.5% homology to the human 5-HT_{1A} sequence, 67.6% to the mouse, 69.2% to tilapia, and 82.4% to pufferfish

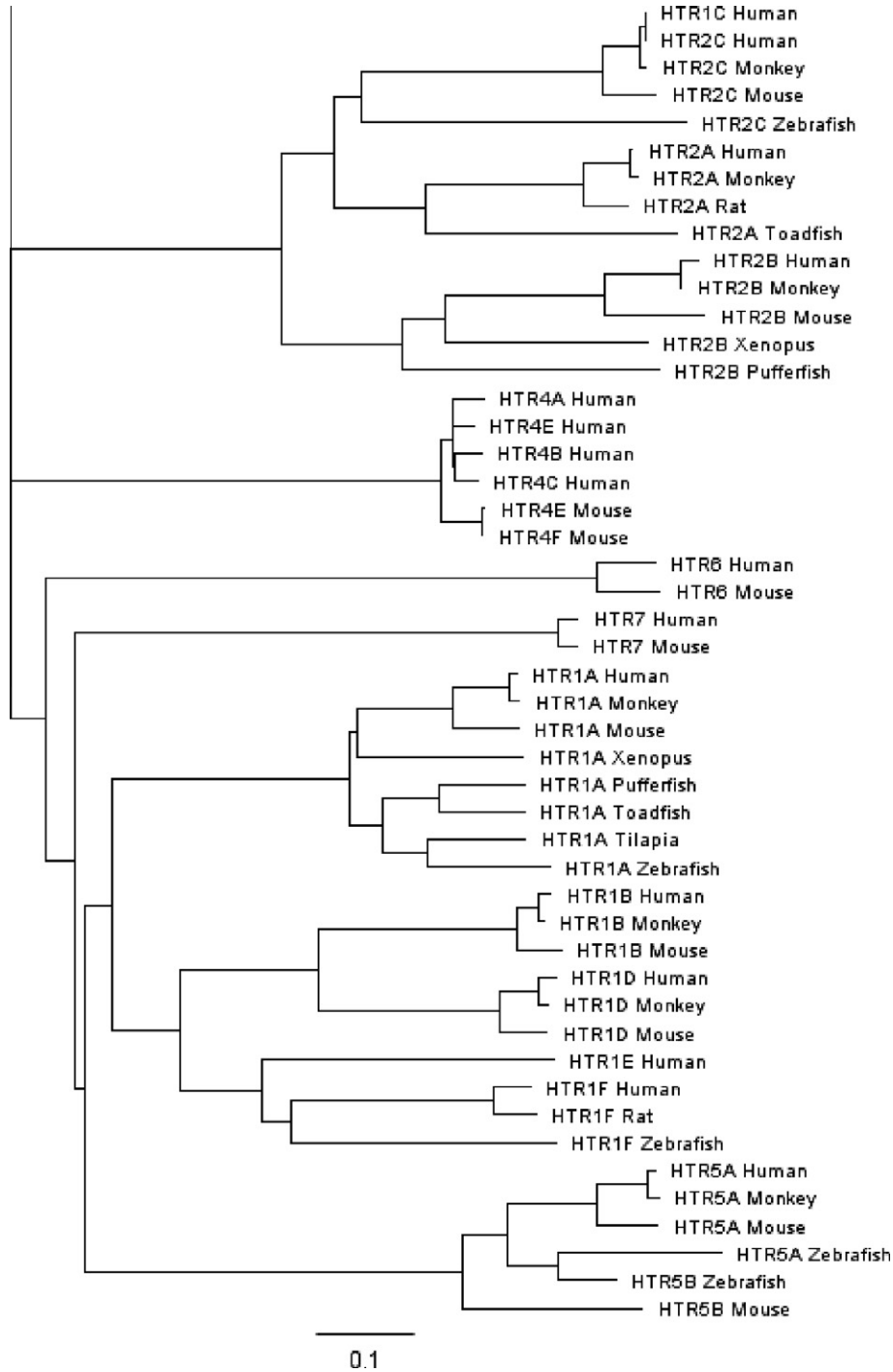


Fig. 2. Phylogenetic analysis of 48 5-HT receptor sequences. Sequences were translated, aligned, and the tree constructed using Geneious Basic (v4.6.4) and the Neighbor Joining method with bootstrap analysis.

(Fig. 1). Phylogenetic analysis of all 5-HT receptors resulted in placing the nucleotide and amino acid sequence for toadfish 5-HT_{1A} in the 5-HT_{1A} receptor branch (Fig. 2). The toadfish sequence had the highest similarity to the pufferfish, but fell out with the other teleosts in the same sub-branch of the 5-HT_{1A} group. Of note, there were two sub-branches in the 5-HT_{1A} grouping, with the teleosts and terrestrial vertebrates separated. Furthermore, the terrestrial vertebrates were separated into amphibians and mammals, validating the sequence analyses. Predicted protein folding using the SOSUI prediction system (<http://bp.nuap.nagoya-u.ac.jp/sosui/>) revealed 7 transmembrane regions with the N-terminus on the extracellular side (Fig. 3).

The 5-HT_{1A} receptor was found to be expressed in many tissues, though predominantly in the brain and reproductive system, as well as the swim bladder (Fig. 4). The brain possessed approximately twofold the amount of 5-HT_{1A} receptor mRNA as the swim bladder, which had the second highest expression of the transcript (Fig. 4A). The gonad had the third highest level of expression, though only about 20% of the transcript measured in the brain; and had a similar amount of expression compared to other tissues (e.g., the posterior intestine, ventral muscle, and rectum) (Fig. 4A). The heart, anterior intestine and kidney, which contain the interrenal cells that are a part of the HPI axis, all contained about 50-fold less 5-HT_{1A} mRNA compared to the whole brain (Fig. 4B). Different regions of the brain (Fig. 5A) expressed the receptor in varying amounts, with the midbrain and diencephalon region having the highest level of 5-HT_{1A} mRNA transcript (Fig. 5B). The midbrain and diencephalon region possessed at least twice as much transcript as other regions and up to 50-fold as much as the cerebellum, which had the lowest expression (Fig. 5B). In regards to the HPI axis, the midbrain and diencephalon region, which contains the hypothalamus and hippocampus, possessed about twice as much 5-HT_{1A} transcript as the pituitary alone.

Xenopus oocytes injected with toadfish 5-HT_{1A} cRNA showed a 1.9-fold increase in [³H]5-HT binding compared to water-injected oocytes when incubated with 1.0×10^{-9} mol l⁻¹ of 5-HT alone (Fig. 6A). The non-specific component of binding was not removed in Figure 6A to demonstrate that the water-injected controls were binding a significantly lower amount of [³H]5-HT than those in-

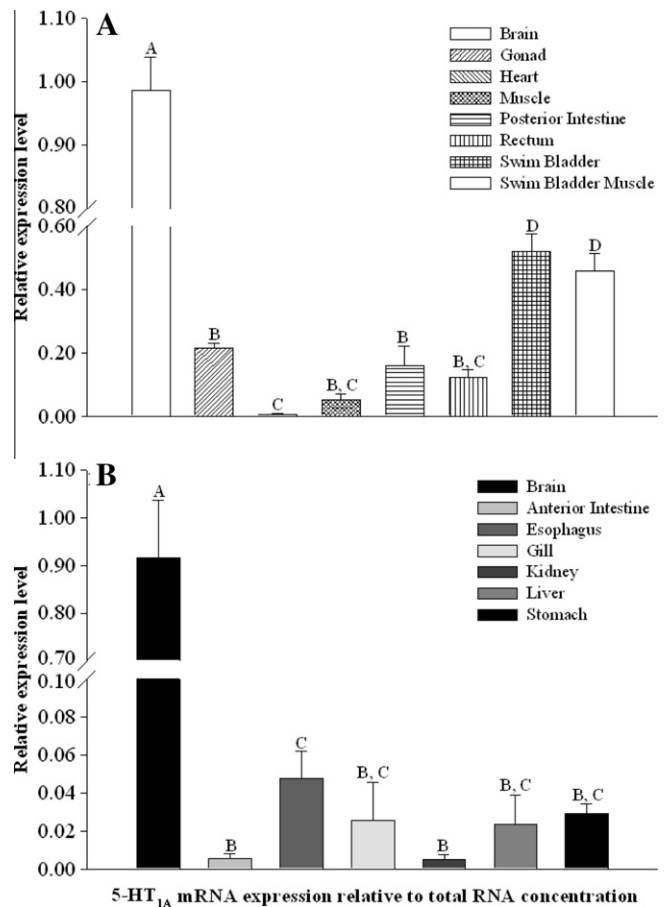


Fig. 4. Relative quantity of 5-HT_{1A} mRNA expression present in various tissues of the Gulf toadfish, *Opsanus beta*. (A) Transcript levels in gonad, heart, muscle, posterior intestine, rectum, swim bladder, swim bladder muscle relative to the brain from one subset of fish ($N = 6$) and (B) transcript levels from anterior intestine, esophagus, gill, kidney, liver and stomach relative to brain from a second set of fish. Values are means \pm s.e.m. A different letter depicts significant difference ($p < 0.05$).

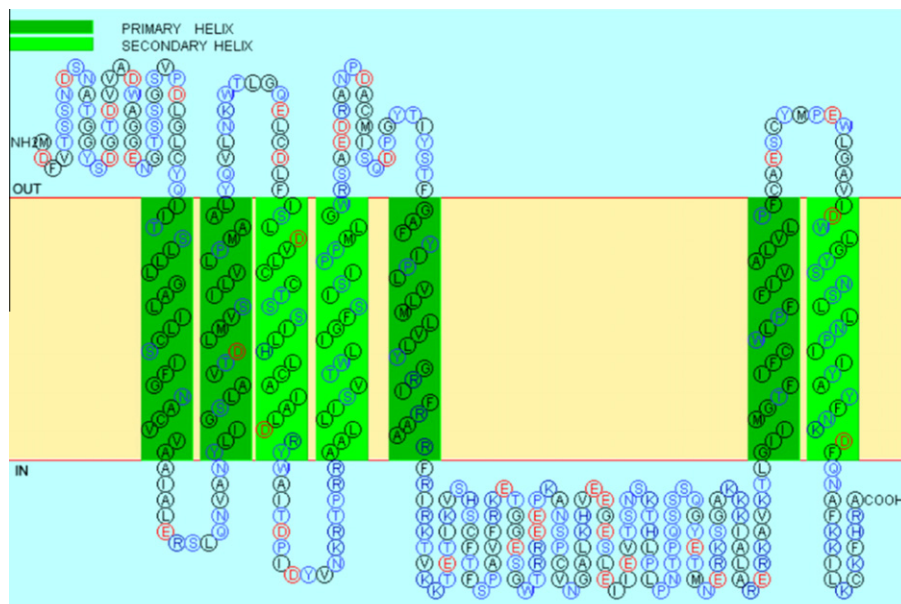


Fig. 3. Predicted amino acid sequence and resulting membrane structure of Gulf toadfish 5-HT_{1A} receptor predicted by the SOSUI prediction system (<http://bp.nuap.nagoya-u.ac.jp/sosui/>).

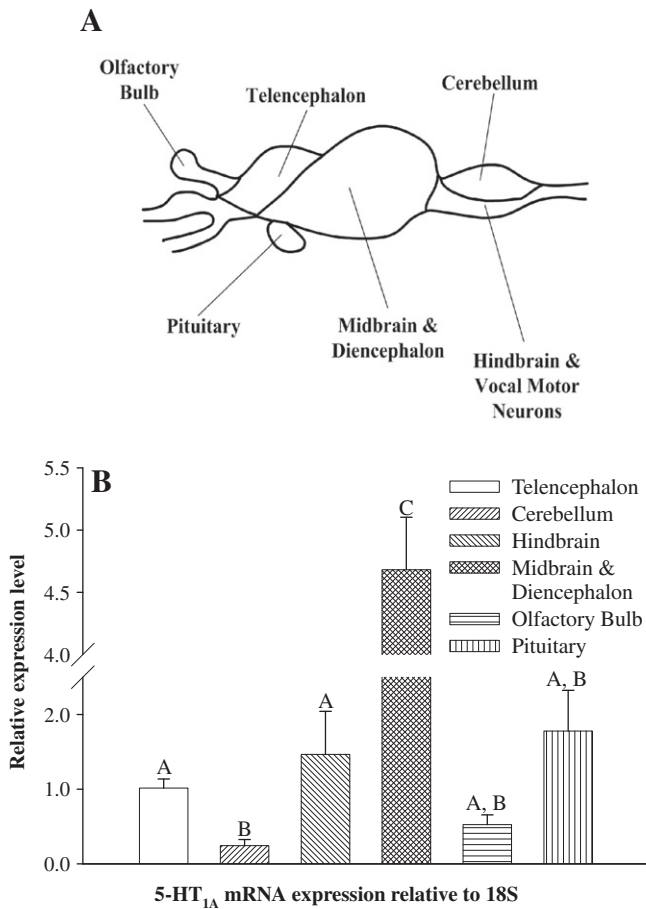


Fig. 5. A. Schematic diagram depicting the way in which the brain was sectioned. Sectioning was intended to separate the brain into the following components: olfactory bulb, telencephalon, pituitary, midbrain & diencephalon, cerebellum, and the medulla & vocal motor neuron region. B. Relative quantity of 5-HT_{1A} mRNA expression present in various regions of the brain, with transcript levels normalized to the housekeeping gene 18S and quantities relative to the telencephalon. Values are means \pm s.e.m., $N = 8$. A different letter depicts significant difference ($p < 0.05$).

jected with 5-HT_{1A} cRNA. For this reason, the binding of [³H]5-HT appears significantly higher than in the subsequent binding experiments (below). A dose-dependent binding curve demonstrating saturation kinetics was established using [³H]5-HT concentrations varying from 0 to 2×10^{-9} mol l⁻¹ ($p < 0.01$, $R^2 = 0.98$; Fig. 6B), giving a B_{max} of 0.1192×10^{-15} mol l⁻¹ ($p < 0.01$) and a K_D of 4.998×10^{-10} mol l⁻¹ ($p < 0.01$). Due to the low K_D value ascertained from the 5-HT binding affinity curve, all subsequent competitive binding experiments used a fixed [³H]5-HT concentration of 1.0×10^{-9} mol l⁻¹. Binding of [³H]5-HT by 5-HT_{1A}-injected oocytes was displaced by the mammalian 5-HT_{1A} receptor agonist, 8-OH-DPAT; a significant regression ($p < 0.01$; $R^2 = 0.98$) and an EC50 value of $2.0\text{--}2 \times 10^{-11}$ mol l⁻¹ ($p = 0.0006$) was obtained for 8-OH-DPAT displacement over the range of $0.0\text{--}1.0 \times 10^{-8}$ mol l⁻¹ 8-OH-DPAT (Fig. 7A). Displacement of [³H]5-HT by agonists or an antagonist specific for other HTR subtypes was investigated using concentrations of pharmacological compounds close to the EC50 value established for 8-OH-DPAT (Fig. 7A), demonstrating significant displacement of [³H]5-HT binding only in oocytes incubated with 8-OH-DPAT, an agonist specific for mammalian 5-HT_{1A} receptors, and not the pharmacological compounds specific for other HTR subtypes (Fig. 7B, 7C).

Intravenous injection with 8-OH-DPAT elicited a concentration-dependent increase in circulating levels of cortisol in the Gulf toadfish (Fig. 8A). When injected with 8-OH-DPAT at a dose of

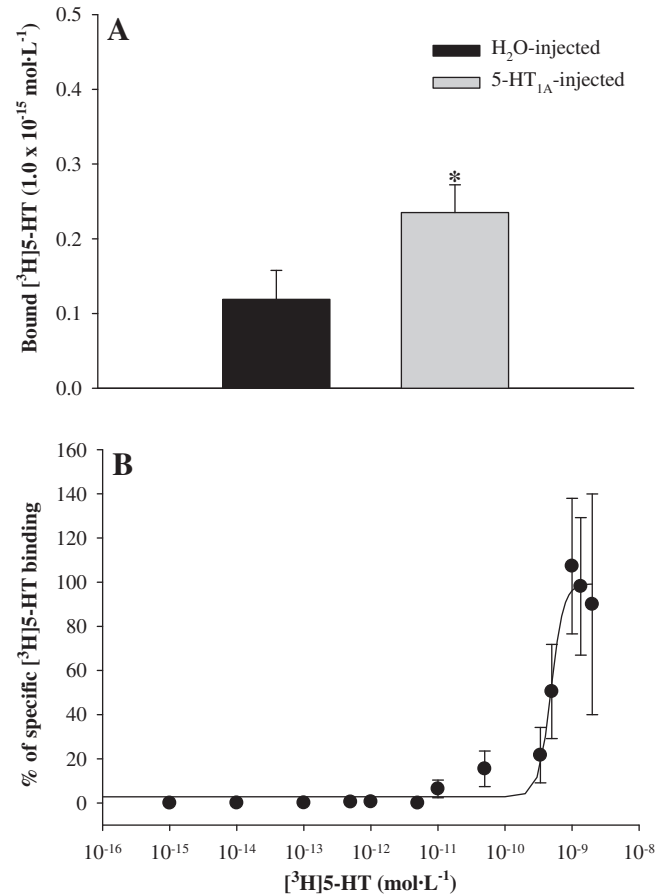


Fig. 6. A. The amount of [³H]5-HT bound to *Xenopus* oocytes injected with toadfish 5-HT_{1A} cRNA as compared to water-injected controls. Concentration of [³H]5-HT was at 1.0×10^{-9} mol l⁻¹. Values are means \pm s.e.m., $N = 10$, * $p < 0.05$. B. The percent binding of [³H]5-HT bound to *Xenopus* oocytes injected with toadfish 5-HT_{1A} cRNA when incubated in a particular concentration of [³H]5-HT ranging from $0.0\text{--}2.0 \times 10^{-9}$ mol l⁻¹. Values are percentages of means relative to 0.0 mol l⁻¹ \pm s.e.m. of the percentage, $N = 10$.

16.25 mg kg⁻¹ ml⁻¹ saline, toadfish experienced a transient, though significant, increase in plasma cortisol levels within 0.25 h that returned to control levels within 4 h (Fig. 8B). While there was a significant increase in plasma cortisol levels, this did not appear to result in a significant gluconeogenesis as circulating levels of glucose, protein or urea were not affected when compared to time zero or controls (Table 2).

4. Discussion

The phylogenetic analysis of the amino acid sequence supports a high degree of homology between the toadfish 5-HT_{1A} receptor and the human homologue, with the toadfish 5-HT_{1A} receptor falling out with the other 5-HT_{1A} amino acid sequences. Additionally, structural analysis of the amino acid sequence revealed a protein that contains 7 transmembrane domains, with an intracellular C terminus and an extracellular N-terminus, which is congruent with the mammalian receptor (Raymond et al., 1999). When comparing the toadfish amino acid sequence to the human sequence, three substitutions become relevant in regards to binding and signaling: Alanine³⁵⁶ in the toadfish is Threonine in humans, Isoleucine³⁶⁴ is Methionine, and Lysine³⁶⁶ becomes Arginine (Raymond et al., 1999). While the switch from Lysine in toadfish to Arginine in humans may not infer any change in binding, as both are α -amino

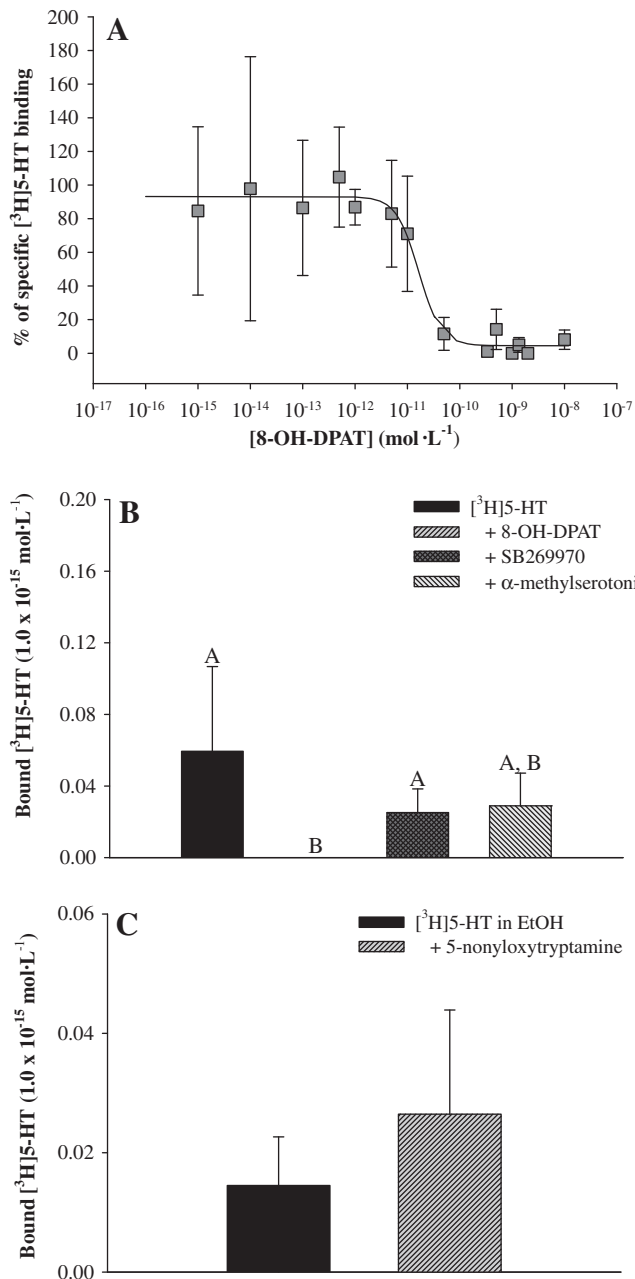


Fig. 7. A. The percent of [^3H]5-HT bound to *Xenopus* oocytes injected with toadfish 5-HT_{1A} cRNA when incubated in the presence of 8-OH-DPAT ranging in concentration from 0.0 to $10.0 \times 10^{-9} \text{ mol l}^{-1}$. The concentration of [^3H]5-HT was $1.0 \times 10^{-9} \text{ mol l}^{-1}$. Values are percentages of means relative to binding at 2.0 nmol l^{-1} , $N = 10$. B. The amount of [^3H]5-HT bound to *Xenopus* oocytes injected with toadfish 5-HT_{1A} cRNA when incubated in the absence or presence of a mammalian 5-HT receptor agonist (8-OH-DPAT, specific for 5-HT_{1A}, and α -methylserotonin, specific for 5-HT₂) or antagonist (SB269970; specific for 5-HT₇). The concentration of [^3H]5-HT was $1.0 \times 10^{-9} \text{ mol l}^{-1}$. Values are means \pm s.e.m., $N = 10$. A different letter depicts a significant difference ($p < 0.05$). C. The amount of [^3H]5-HT bound to *Xenopus* oocytes injected with toadfish 5-HT_{1A} cRNA when incubated in the absence or presence of the mammalian 5-HT_{1B} receptor agonist 5-nonyloxytryptamine in 0.01% ethanol. 0.01% ethanol + [^3H]5-HT alone was used as a vehicle control. The concentration of [^3H]5-HT was $1.0 \times 10^{-9} \text{ mol l}^{-1}$. Values are means \pm s.e.m., $N = 10$. A different letter depicts a significant difference ($p < 0.05$).

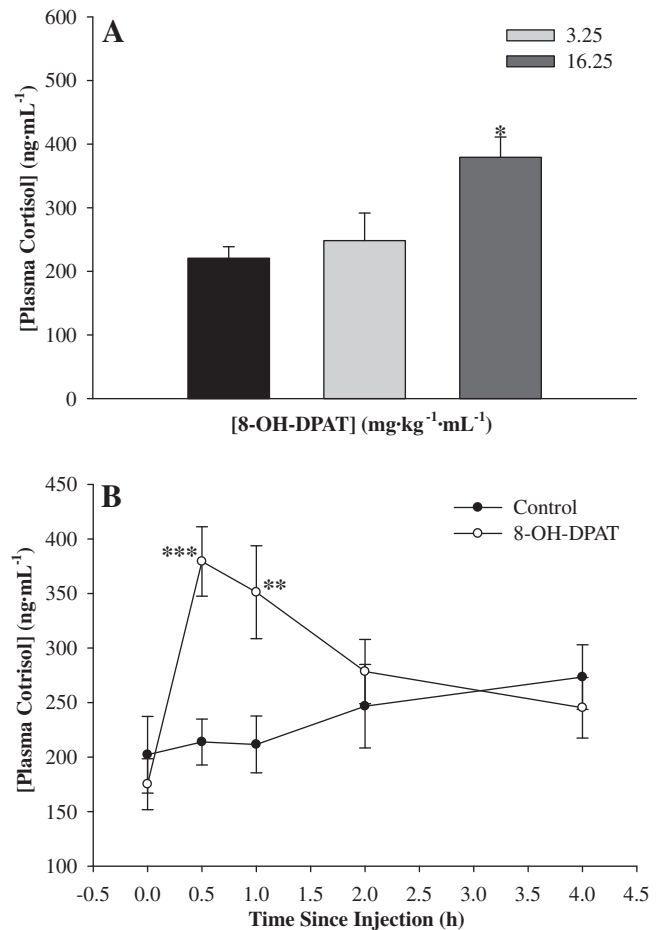


Fig. 8. A. There is a concentration-dependent increase in circulating levels of cortisol in response to injection with 8-OH-DPAT. B. Changes in circulating levels of cortisol over time in response to injection with $16.25 \text{ mg kg}^{-1} \text{ ml}^{-1}$ saline 8-OH-DPAT. Controls are injected with saline alone. Values are means \pm s.e.m., $N = 9$, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

may increase the number of sites available for phosphorylation or glycosylation (Brimer and Montie, 1998). Based on these inferences, most, if not all, of the function of the toadfish 5-HT_{1A} receptor should be conserved; however, the change in amino acids may result in a change in the affinity constant or maximal binding of the receptor. While it would be interesting to compare the binding parameters between the two species to see if this prediction holds true, unfortunately the methods used in human studies and the resulting parameters vary so greatly that it prevents making a scientifically valid comparison.

Consistent with tissue distribution studies investigating 5-HT_{1A} receptor mRNA expression in mammals (Kirchgessner et al., 1993; Raymond et al., 1993; Engel et al., 2006), the basic conclusion from the distribution of 5-HT_{1A} in toadfish is that the brain possesses up to 50-times more 5-HT_{1A} mRNA transcript than other tissues, including the kidney, in which are the interrenal cells, an integral part of the HPI axis. While there is significantly more 5-HT_{1A} mRNA present in the brain than in kidney, it is of note that there appears to be 5-HT_{1A} receptors present in all portions of the HPI axis. The reduced expression of 5-HT_{1A} receptors in the toadfish kidney is in agreement with mammalian studies that suggest that 5-HT_{1A} receptors do not play a role in stimulating the release of cortisol from the adrenal gland, the mammalian homologue to the teleost interrenal cells (Lefebvre et al., 1992; Contesse et al., 1994); however, as the whole kidney was used in the present study and not just isolated interrenal

acids and basic, the other two substitutions most likely confer a difference. Switching Isoleucine to Methionine within the G-protein binding motif may confer an increase in stability of the protein under certain coiling schemes (Garcia-Echeverria, 1997), and substituting Threonine for Alanine at the protein kinase C site

Table 2

Concentrations of circulating compounds in the plasma pre- and post-injection with 8-OH-DPAT or saline. Numbers reported as means \pm s.e.m., $N = 6$, no significant differences between times or treatments.

Time (h)	Control			8-OH-DPAT		
	Glucose (mmol l ⁻¹)	Protein (g 100 ml ⁻¹)	Urea (mmol N l ⁻¹)	Glucose (mmol l ⁻¹)	Protein (g 100 ml ⁻¹)	Urea (mmol N l ⁻¹)
0	1.98 \pm 0.30	1.19 \pm 0.15	9.78 \pm 1.68	2.48 \pm 0.46	1.27 \pm 0.10	9.66 \pm 1.65
0.5	1.73 \pm 0.47	1.24 \pm 0.19	6.18 \pm 2.9	2.59 \pm 1.46	0.86 \pm 0.45	4.42 \pm 1.14
1	1.62 \pm 0.05	1.22 \pm 0.05	8.56 \pm 0.23	3.47 \pm 1.63	1.13 \pm 0.34	10.36 \pm 3.20
2	2.04 \pm 0.82	1.35 \pm 0.11	9.88 \pm 4.39	1.34 \pm 0.34	1.22 \pm 0.19	14.14 \pm 4.22
4	0.63 \pm 0.24	0.92 \pm 0.29	15.59 \pm 2.20	0.56 \pm 0.10	1.30 \pm 0.06	9.47 \pm 2.18

cells, the relative mRNA expression of the toadfish 5-HT_{1A} by the interrenal cells is likely underestimated. Furthermore, renal 5-HT_{1A} protein function was not evaluated in the present study, and since mRNA expression does not always represent protein activity (McDonald et al., submitted for publication), it cannot be concluded that 5-HT_{1A} receptors in the interrenal cells are not playing a substantial role. Surprisingly, the toadfish swim bladder and swim bladder muscle also possess high amounts of 5-HT_{1A} receptor. The toadfish is so named for the croaking noises it emits when agitated, but the males are also able to produce a boatwhistle call when attracting mates to potential nesting sites (Serafy et al., 1997; Goodson and Bass, 2002). Sound production occurs as a result of the swim bladder muscle rapidly contracting against the swim bladder membrane, a movement that is controlled by the vocal motor neurons (Goodson and Bass, 2002), located in the hindbrain where moderate amounts of 5-HT_{1A} receptor is expressed. Recent work investigating 5-HT_{1A} receptor function in mammals and fish has focused on the involvement of the receptor in reproduction and associated behaviors (Wada et al., 2006; Smith and Combs, 2008), supporting a potential role of the 5-HT_{1A} receptor in toadfish, potentially in the modulation of sound production that is part of their courting behavior.

Studies conducted in the rat and human have found that the 5-HT_{1A} receptor is widely distributed throughout the brain, though the amount of mRNA and/or binding activity varies greatly depending on the region being addressed (Chalmers and Watson, 1991; Pompeiano et al., 1992). In mammals, particularly high expression of 5-HT_{1A} is found within the hippocampus, pituitary, and raphe nuclei whereas the cerebellum, amygdala, corpus striatum and globus pallidus have especially low levels (Passchier et al., 2000; Kumar and Mann, 2007). In terms of function, the hippocampus is important in providing negative feedback on the HPA axis while the pituitary is paramount to most endocrine functions, including the regulation of the HPA axis. The raphe nuclei is a major source of brain 5-HT and contains the somatodendritic 5-HT_{1A} autoreceptors, which play a role in controlling the release of 5-HT in other areas of the CNS. Distribution of 5-HT_{1A} transcript throughout the brain varied in toadfish greatly, though the expression pattern is congruent with the mammalian studies. The toadfish midbrain and diencephalon section, which includes the pallial region (analogous to the hippocampus in higher vertebrates) and the rostral raphe nuclei, contained a significantly higher level of 5-HT_{1A} mRNA transcript than all other regions. Given the relatively high conservation of the serotonergic system in vertebrates, it is likely that the high expression of 5-HT_{1A} receptors found in this region is due to the rostral raphe nuclei and pallial region, as the 5-HT_{1A} receptor is present only in low levels in the hypothalamus of humans and other mammals (Passchier et al., 2000; Kumar and Mann, 2007). The relatively high amounts of 5-HT_{1A} mRNA transcript in the toadfish pituitary is consistent to what is found in mammals (Chalmers and Watson, 1991; Lopez et al., 1998; Passchier et al., 2000; Kumar and Mann, 2007), not surprising if the role of 5-HT in the regulation of the stress response is conserved across taxa.

Supporting the apparent conservation of function across taxa, 5-HT_{1A}-injected *Xenopus* oocytes incubated with 1.35×10^{-9} mol l⁻¹ [³H]5-HT, the predicted K_D for the population of 5-HT receptors in toadfish brain determined using a whole-brain homogenate preparation (LR Medeiros and MD McDonald, unpublished observation), had significantly higher [³H]5-HT binding compared to the water-injected oocytes. Furthermore, 5-HT_{1A}-injected *Xenopus* oocytes incubated with [³H]5-HT and equal amounts of 8-OH-DPAT exhibited significantly lower binding as compared to those incubated with [³H]5-HT alone, indicating that the toadfish 5-HT_{1A} receptor binds both 5-HT and the mammalian 5-HT_{1A} agonist, 8-OH-DPAT. That 8-OH-DPAT was able to completely eliminate 5-HT binding when present in equal concentrations suggests that the toadfish 5-HT_{1A} receptor has a higher affinity for 8-OH-DPAT than for 5-HT, a trend that is also seen in the mammalian 5-HT_{1A} receptor (Dompert et al., 1985; Hoyer et al., 1985; Peroutka, 1986). This assumption was confirmed when performing the 5-HT and 8-OH-DPAT dose-dependent binding curve, when the K_D for 5-HT binding (4.998×10^{-10} mol l⁻¹) was determined to be about half an order of magnitude higher than the EC₅₀ determined for 8-OH-DPAT ($2.0\text{--}2 \times 10^{-11}$ mol l⁻¹). This is in accordance with the findings observed in studies on other species of fish as well as mammals (Winberg and Nilsson, 1996; Zifa and Fillion, 1992). That all other subtypes' agonists and antagonists were unable to abolish the binding of 5-HT further supports that the toadfish 5-HT_{1A} receptor has very similar, if not identical, binding sites as the mammalian counterpart. Interestingly, the concentration of 8-OH-DPAT used in this set of experiments (1.8×10^{-11} mol l⁻¹; Fig. 7B) was able to completely eliminate binding of [³H]5-HT. This concentration is slightly lower than the established EC₅₀ (Fig. 7B cf. Fig. 7A); the regression obtained from the EC₅₀ curve predicts that about 55% of binding would still be present at this concentration of 8-OH-DPAT. The most likely explanation for the anomaly is that the slope of the curve at this concentration is extremely steep, and therefore contains more inherent variation at these concentrations than at either end of the range.

The similarity between the toadfish 5-HT_{1A} receptor and mammalian homologue in terms of amino acid sequence, mRNA distribution within the HPI axis and pharmacological sensitivity was further supported by functional similarity on the whole animal level. Injection with the mammalian 5-HT_{1A} agonist 8-OH-DPAT caused a significant increase in plasma cortisol levels that returned to control levels within 4 hours. The endocrine response to 8-OH-DPAT injection, combined with the *Xenopus* oocyte data, indicates a specific, potent effect mediated by the 5-HT_{1A} receptor in the Gulf toadfish. The timing of the response is consistent with other teleost fish (Zifa and Fillion, 1992; Winberg et al., 1997; Hoglund et al., 2002). For example, rainbow trout (*Oncorhynchus mykiss*) injected with a 0.040 mg kg⁻¹ dose of 8-OH-DPAT experienced a significant increase in plasma cortisol levels within 30 min that returned to control levels after 4 h (Winberg et al., 1997). The higher dose of 8-OH-DPAT needed to elicit a response in toadfish (16.25 mg kg⁻¹) may be due to differences in ambient water temperature (see Sundin et al., 1998) or a species-specific response to 8-OH-DPAT. In

any case, the observed increase in and subsequent metabolism of plasma cortisol in toadfish in response to 8-OH-DPAT is similar to what is observed in mammals when injected with the same compound (Zifa and Fillion, 1992), indicating that, in addition to a molecular and pharmacological consistency between the 5-HT_{1A} receptor of mammals and toadfish, there is also a functional similarity in terms of its role in the regulation of the stress response. Based on our results from the *in vitro* oocyte binding assays, it is unlikely that the increase in circulating levels of cortisol, due to activation of 5-HT_{1A}, could be mediated by another 5-HT receptor agonist or antagonist. However, we have not eliminated the possibility that 8-OH-DPAT may be activating other receptors in this non-mammalian system that are involved in stimulating cortisol release (i.e., 5-HT₄ receptors; Lefebvre et al., 1992; Contesse et al., 1994), which could be contributing to the observed *in vivo* response.

This investigation provides the first look at 5-HT_{1A} receptor molecular characteristics, tissue distribution, pharmacology and neuroendocrine function in a single teleost fish species. The findings suggest a high conservation of the 5-HT_{1A} receptor amongst vertebrates and validate the Gulf toadfish as a potential model organism for the study of vertebrate 5-HT_{1A} receptor function. Due to the emerging evidence concerning the presence and opposing functionality of the two 5-HT_{1A} receptor populations (somatodendritic autoreceptors in the raphe nucleus and postsynaptic receptors elsewhere in the brain and periphery; Lanfumey and Hamon, 2000), future investigations would benefit from utilizing recently developed agonists that differentiate between the two populations in order to determine a clear role for each in terms of HPI axis regulation. Only by increasing our knowledge of 5-HT_{1A} receptor function can we begin to understand the more complicated physiological and behavioral systems with which it is involved.

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