

RESEARCH ARTICLE

Molecular and functional characterization of the Gulf toadfish serotonin transporter SLC6A4

Molly H. B. Amador* and M. Danielle McDonald

ABSTRACT

The serotonin transporter (SERT) functions in the uptake of the neurotransmitter serotonin (5-HT) from the extracellular milieu and is the molecular target of the selective serotonin re-uptake inhibitors (SSRIs), a common group of anti-depressants. The current study comprehensively assesses the sequence, tissue distribution, transport kinetics and physiological function of a teleost SERT. The 2022 bp toadfish SERT sequence encodes a protein of 673 amino acids, which shows 83% similarity to zebrafish SERT and groups with SERT of other teleosts in phylogenetic analysis. SERT mRNA is ubiquitous in tissues and is expressed at high levels in the heart and, within the brain, in the cerebellum. SERT cRNA expressed in *Xenopus laevis* oocytes demonstrates a K_m value of $2.08 \pm 0.45 \mu\text{mol l}^{-1}$, similar to previously reported K_m values for zebrafish and human SERT. Acute systemic blockade of SERT by intraperitoneal administration of the SSRI fluoxetine (FLX) produces a dose-dependent increase in plasma 5-HT, indicating effective inhibition of 5-HT uptake from the circulation. As teleosts lack platelets, which are important 5-HT sequestration sites in mammals, the FLX-induced increase in plasma 5-HT suggests that toadfish tissues may normally be responsible for maintaining low 5-HT concentrations in the bloodstream.

KEY WORDS: 5-HT, 5-Hydroxytryptamine, 5-HTT, Transport kinetics, mRNA expression, *Opsanus beta*

INTRODUCTION

Serotonin (5-hydroxytryptamine; 5-HT) is a monoamine signaling molecule influencing a wide array of behaviors and physiological processes in vertebrates, controlling heart rate, vascular resistance, respiration, the stress response, digestion, reproduction and other processes (reviewed in Berger et al., 2009). 5-HT acts as a neurotransmitter in the central nervous system; however, it is also extensively utilized in the periphery as a paracrine or endocrine signal (reviewed in Berger et al., 2009; Pelster and Schwerte, 2012; El-Merahbi et al., 2015). The 5-HT transporter (SERT; SLC6A4) plays an important role in the signaling process. SERT removes 5-HT from the synapse or extracellular space – effectively terminating the signal and allowing the 5-HT to be stored, recycled or degraded intracellularly – and is largely known as the target of the selective serotonin re-uptake inhibitors (SSRIs), a common class of anti-depressants including drugs such as fluoxetine (FLX; reviewed in Fuller and Wong, 1990; Ni and Watts, 2006; Berger et al., 2009).

In mammals, SERT has been found to be a membrane-bound Na^+ - and Cl^- -dependent solute carrier with 12 putative transmembrane domains encoded by mRNA transcripts approximately 1.8–2.3 kb in length, which are translated into primary protein structures of approximately 600–630 amino acids (Blakely et al., 1991; Ramamoorthy et al., 1993; Chang et al., 1996; Chen et al., 1998a,b; Mortensen et al., 1999). Mammalian SERT is widely distributed throughout the body. Within the brain, SERT transcript and protein have been detected in numerous brain regions, most notably the raphe nuclei of the midbrain (Blakely et al., 1991; Duncan et al., 1992; Lesch et al., 1993a; Chang et al., 1996; Kish et al., 2005). In the rest of the body, SERT has been documented in cardiovascular, respiratory, urinary, endocrine, skeletal and digestive system tissues (Mortensen et al., 1999; Linder et al., 2009). SERT is also found on mammalian platelet plasma membranes, where it enables sequestration and storage of vasoactive 5-HT from the bloodstream (reviewed by Ni and Watts, 2006).

SERT appears to be well conserved among vertebrates, including teleost fish, which are increasingly used as model organisms. It has been sequenced in several teleost fish but has only been comprehensively described in zebrafish, in which two SERT-encoding genes have been identified: *serta* and *sertb* (proteins: SERTA and SERTB; Wang et al., 2006; Severinsen et al., 2008). At a functional level, fish SERTs appear to possess a lower affinity for 5-HT than mammalian SERTs (Wang et al., 2006; Severinsen et al., 2008), although they may be inhibited by SSRIs with similar effectiveness (Wang et al., 2006; Severinsen et al., 2008; reviewed by McDonald, 2017). SERT mRNA transcript has been documented both within the central nervous system and in peripheral tissues of teleost fish (Wang et al., 2006; Norton et al., 2008; Mennigen et al., 2010a), where it could be involved in such 5-HT-sensitive processes as catecholamine and cortisol secretion (Fritsche et al., 1993; Bernier and Perry, 1996; Medeiros and McDonald, 2012; Lim et al., 2013) or branchial (Fritsche et al., 1992; Sundin, 1995; Sundin et al., 1995; Forster et al., 1998; Nilsson and Sundin, 1998; McDonald et al., 2010), gastrointestinal (Kiliaan et al., 1989; Mori and Ando, 1991; Venugopalan et al., 1995; Buddington and Krogdahl, 2004; Velarde et al., 2010) or cardiovascular (Janvier et al., 1996; Pellegrino et al., 2003; McDonald et al., 2010) functions. Interestingly, teleost thrombocytes, the evolutionary precursors of platelets – which are the primary circulating 5-HT storage pools in mammals (Mercado and Kilic, 2010) – do not appear to play a 5-HT storage role in teleosts. Teleost lymphocytes (like their mammalian counterparts; Marazziti et al., 1998; Barkan et al., 2004) do demonstrate SSRI-sensitive 5-HT uptake (Ferriere et al., 1999); however, 5-HT concentrations in whole blood of rainbow trout are similar to those in plasma alone, suggesting that most, if not all, 5-HT circulates extracellularly (Maurer-Spurej, 2005; Caamaño-Tubío et al., 2007). It is therefore possible that another peripheral SERT location –

Department of Marine Biology and Ecology, Rosenstiel School of Marine and Atmospheric Science, University of Miami, Miami, FL 33149, USA.

*Author for correspondence (mbroome@rsmas.miami.edu)

 M.H.B.A., 0000-0003-0621-3528

Received 22 September 2017; Accepted 19 February 2018

perhaps a tissue – is important in controlling 5-HT in the bloodstream. Overall, the roles of 5-HT in the teleost periphery remain only superficially understood and, by extension, so do the peripheral functions of SERT.

Fully understanding SERT and other aspects of 5-HT signaling in teleosts is critical as SSRIs are increasingly found as pollutants in aquatic environments, where they can potentially interfere with 5-HT signaling in exposed organisms (Kolpin et al., 2002; Brooks et al., 2005; Barnes et al., 2008; Ramirez et al., 2009; reviewed in Brooks et al., 2003; Silva et al., 2015; McDonald, 2017). Furthermore, understanding the characteristics and functions of SERT earlier in evolution will enhance understanding of its role in mammals. Therefore, the objectives of the present study were to sequence the Gulf toadfish SERT; to compare it with SERT of other organisms; to examine its tissue distribution; to characterize its transport kinetics and ion dependence; and to examine the consequences of systemic SERT inhibition by FLX on toadfish plasma 5-HT dynamics over time. We hypothesized that toadfish SERT would be broadly expressed across tissues and would show similar transport characteristics to other teleost SERTs. We also hypothesized that there would be dose-dependent increases in plasma 5-HT after FLX treatment that may reflect the inhibition of SERT-mediated 5-HT sequestration in the periphery.

MATERIALS AND METHODS

Experimental animals

Gulf toadfish, *Opsanus beta* (Goode & Beane 1880), were caught as roller trawl bycatch by local shrimpers in Biscayne Bay, Florida during the winter and spring of 2014/2015 (Florida Fish and Wildlife Conservation Commission Special Activity License no. SAL-12-0729-SR). Upon their arrival in the laboratory, fish were treated with final concentrations of 0.05 mg l⁻¹ malachite green and 15 mg l⁻¹ formalin (ProForm-C; Koi Care Kennel, Westminster, CA, USA) to treat and prevent infection by ectoparasites. Fish were housed in aerated flow-through 20-gallon aquaria supplied with filtered seawater from Biscayne Bay and were fed shrimp weekly to satiation. Water temperatures ranged from 20 to 24°C. All experimental protocols involving animals were approved by the University of Miami Institutional Animal Care and Use Committee.

Tissue collection, RNA isolation, DNase treatment and cDNA synthesis

Fish were anesthetized in buffered 1 g l⁻¹ tricaine methanesulfonate (MS-222, pH 8.2; Western Chemical, Ferndale, WA, USA). Whole tissues (brain, heart, gill arches, esophagus, stomach, anterior intestine, mid-intestine, posterior intestine, rectum, liver, spleen and kidney) as well as brain segments (olfactory bulb, telencephalon, pituitary gland, midbrain and diencephalon, cerebellum and hindbrain; as described by Medeiros et al., 2010) were harvested, flash-frozen in liquid nitrogen and stored at -80°C until RNA isolation. At a later date, samples of whole blood were collected by caudal puncture with 23-gauge needles attached to syringes that had been rinsed with heparinized saline (50 IU ml⁻¹); another group of whole brain samples was also collected, and RNA was isolated from these blood and brain samples immediately (without freezing). For RNA isolation, TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) was added to tissues and blood in a 6:1 ratio. Tissues were homogenized with an IKA T 10 basic ULTRA-TURRAX homogenizer (IKA Works, Staufen im Breisgau, Germany) and blood samples were vortexed thoroughly. Samples were then phase-separated with chloroform, and RNA was precipitated with

isopropanol as described in the Trizol reagent accompanying protocol. RNA was resuspended in molecular biology grade water (Sigma-Aldrich, St Louis, MO, USA) and quantified using a SpectraMax Plus 384 plate reader and SpectraDrop Micro-Volume Microplate with Softmax Pro software (Molecular Devices, Sunnyvale, CA, USA).

To remove any contaminating DNA, 10 µg of each RNA sample was DNase-treated using the Turbo DNA-free Kit (Thermo Fisher Scientific); 0.8 µg of each DNA-free sample was then transcribed into cDNA with the SuperScript III First Strand Synthesis System for reverse transcription polymerase chain reaction (RT-PCR) (Thermo Fisher Scientific) using random hexamers.

Full-length sequence and phylogenetic analysis

Custom primers (Table 1) were designed based on the toadfish transcriptome (Schauer et al., 2017) and used in PCR with the Advantage 2 PCR Kit (Takara Bio USA, Mountain View, CA, USA) on a PTC-200 thermocycler (MJ Research, Waltham, MA, USA). PCR products were gel-purified using the QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned using the TOPO TA Cloning Kit for Sequencing (Thermo Fisher Scientific). Plasmids were then isolated using the QIAprep Spin Miniprep Kit (Qiagen) and Sanger sequenced (Genewiz, South Plainfield, NJ, USA). Pairwise alignments (KAlign), multiple alignments (MUSCLE) and phylogenetic analyses (PhyML) were performed in UGene software (Unipro, Novosibirsk, Russia). Protein secondary structures were predicted using MEMSAT2 (Jones et al., 1994).

Toadfish SERT expression in *Xenopus laevis* oocytes

Molecular preparation

The full-length SERT insert was excised and ligated into the *EcoRI* site of the pGH19 *Xenopus laevis* oocyte expression vector (a kind gift from Dr Zhirong Jiang, Yale University School of Medicine, New Haven, CT, USA) using T4 DNA ligase (Promega, Madison, WI, USA); the ligation reaction was then used to transform JM109 competent cells (Promega). Subsequently, isolated plasmids were screened for proper insert orientation by sequencing. Plasmids with properly oriented inserts were linearized using high-fidelity *NheI* (*NheI*-HF; New England Biolabs, Ipswich, MA, USA), treated with Proteinase K (New England Biolabs), extracted with phenol:chloroform:IAA (Sigma-Aldrich), and precipitated overnight with 100% ethanol. Pellets were re-suspended in molecular biology grade water, DNA was quantified, and 1 µg was used as a template for cRNA transcription. cRNA was synthesized using the mMESSAGE mMACHINE T7 Kit (Thermo Fisher Scientific) as per the manufacturer's instructions, quantified, and adjusted to a concentration of approximately 1 µg µl⁻¹ with nuclease-free water in preparation for injection into oocytes.

Oocyte injection and maintenance

Collagenase-treated, defolliculated oocytes (a kind gift from Dr Gerhard Dahl, University of Miami, Miller School of Medicine,

Table 1. Primers used in cloning and qPCR of the toadfish SERT

Primer	Sequence (5'→3')
SERT forward	GGCACATTCAAACAGCGTC
SERT reverse	AGATGATGGTGCTCTGCG
SERT forward qPCR	GGCACATTCAAACAGCGTC
SERT reverse qPCR	AGATGATGGTGCTCTGCG

Miami, FL, USA) were maintained in antibiotic-free OR2 medium (mmol l⁻¹: 82.5 NaCl, 2.5 KCl, 1 CaCl₂, 1 MgCl₂, 1 Na₂HPO₄, 5 Hepes; pH 7.8, autoclaved; Medeiros et al., 2010; Taylor et al., 2010) at 17°C until time of injection. Oocytes were microinjected with 50.6 ng (~1 ng nl⁻¹) cRNA or equivalent volume of MilliQ water (control group) using a Nanoliter 2000 Injector (World Precision Instruments, Sarasota, FL, USA). Post-injection, oocytes were maintained in ND96 medium (mmol l⁻¹: 99 NaCl, 2 KCl, 1.8 CaCl₂, 1 Hepes, 1 MgCl₂; pH 7.4, autoclaved; Taylor et al., 2010) supplemented with antibiotics (10,000 IU ml⁻¹ penicillin, 10 mg ml⁻¹ streptomycin) at 17–19°C until assays were performed. Immediately before all assays, oocytes were rinsed twice in antibiotic-free ND96 medium.

Experimental series

After rinsing, water-injected and cRNA-injected oocytes were added in parallel to wells of a 96-well conical bottom plate with a disposable plastic pipette; oocytes were allowed to settle to the bottom of the pipette before adding to minimize the addition of medium to the wells. Each well contained 150 µl of a bath solution of non-radioactive 5-HT (as 5-HT hydrochloride; Sigma-Aldrich) and [³H]5-HT (as [³H]5-HT creatinine sulfate, 27.7 Ci mmol⁻¹; American Radiolabeled Chemicals, St Louis, MO, USA) in antibiotic-free ND96 medium. To determine the optimal post-cRNA injection waiting period and assay incubation times for experiments, uptake assays (Fig. S1) were performed with oocytes 48, 72 or 96±2 h after cRNA injection and with assay incubation times of 0, 2.5, 5, 10, 20 or 40 min at a final 5-HT concentration of 2 µmol l⁻¹; this concentration was chosen based on the *K_m* (Michaelis constant) value of zebrafish SERT, estimated to be 2.13 and 4.2 µmol l⁻¹ by Severinsen et al. (2008) and Wang et al. (2006), respectively. Based on these preliminary trials, a post-injection time of 72 h and an assay incubation time of 5 min was used for all experiments. To measure toadfish SERT transport kinetics, bath solutions were prepared with seven different concentrations of [³H]5-HT diluted with non-radioactive 5-HT to create a concentration series of 0.0361, 0.3, 0.6, 1, 3, 6 and 10 µmol l⁻¹ total 5-HT. To determine the extent of Na⁺ and Cl⁻ dependence, bath solutions that excluded each ion were tested. Both dependence assays contained 2 µmol l⁻¹ total 5-HT supplemented with [³H]5-HT. In Na⁺ dependence assays, NaCl in ND96 medium was replaced with equimolar *N*-methyl-D-glucamine-Cl; in Cl⁻ dependence assays, chloride salts were replaced with gluconate salts.

For each experimental series, a 10 µl sample of the assay solution was taken for liquid scintillation counting. After the 5 min incubation period, oocytes were rinsed three times in ice-cold antibiotic-free ND96 medium containing non-radioactive 5-HT at 10 times the concentration of the bath solution to stop uptake. In the transport kinetics experiment, each rinse volume was 300 µl; after washing, oocytes from each well were divided among three translucent plastic liquid scintillation vials (three replicates, two to six oocytes per replicate) containing 1 ml of 10% SDS and allowed to dissolve overnight or longer. In the Na⁺ and Cl⁻ dependence experiments, rinse volumes were 2 ml and included 2.86 µmol l⁻¹ of the SERT inhibitor FLX (as fluoxetine hydrochloride; Sigma-Aldrich) to minimize the possibility of reverse transport in wash solutions. Furthermore, to reduce photon quenching by oocyte yolk (Gerhard Dahl, University of Miami, personal communication) and thus to improve beta counting resolution, oocytes were not dissolved in SDS. Instead, replicates of two to six oocytes were placed in 1.5 ml microcentrifuge tubes, 10–15 µl of water was added and

tubes were flicked to lyse the oocytes. Samples were then centrifuged for 5–10 min at 16,100 *g*, and 10 µl of each supernatant was added to scintillation vials.

A volume of 10 ml of EcoLume liquid scintillation cocktail (MP Biomedicals, Santa Ana, CA, USA) was added to each scintillation vial containing bath solution, dissolved oocytes or oocyte supernatant. Vials were shaken vigorously and sat overnight in the dark before radioactivity was counted for 5 min in a Wallac 1415 liquid scintillation counter (Wallac Oy, Turku, Finland) using MultiCalc software (version 1.52; Wallac Oy) with no quench correction. The specific activities of the bath solutions in each well were calculated based on the measured radioactivity (as counts per minute, c.p.m.) and the 5-HT concentration of the bath solution in each well. The specific activity and incubation time for each well were then used to convert oocyte radioactivity measurements into rates of 5-HT uptake. Non-specific binding was removed by subtracting the mean uptake in water-injected oocytes from the uptake in corresponding cRNA-injected oocytes.

SERT mRNA distribution analysis with quantitative PCR

To analyse SERT transcript distribution among a suite of tissues, quantitative PCR (qPCR) was performed using 10-fold dilutions of cDNA in molecular biology grade water (Sigma-Aldrich), gene-specific primers (Table 1), and POWER SYBR Green PCR Master Mix as a reporter dye (Thermo Fisher Scientific) on an Mx3005P Multiple Quantitative PCR System (Stratagene, La Jolla, CA, USA) with MxPro Software (version 4.10; Stratagene). The cycling parameters were as follows: 95°C for 10 min; 50 cycles of 95°C for 30 s, 61°C for 30 s, 72°C for 30 s; and 95°C for 1 min. A dissociation curve was created from 60 to 95°C in 1 min increments to verify the formation of only one amplification product. No-reverse-transcriptase and no-template controls were run to ensure the absence of contaminating DNA and primer dimers, respectively. Calibrator samples (identical aliquots of a mix of cDNA from multiple tissues and multiple fish) were run on each plate to allow comparison of results across plates. Samples were run in duplicate; the mean of duplicate runs was used for further analysis. In cases where only one replicate produced a *C_t* value, that *C_t* value was used. Data were analysed using the 2^{-ΔΔ*C_t*} method as described by Livak and Schmittgen (2001). EF1A, 18S and GAPDH were considered as potential reference genes; however, expression of these genes varied across tissues and precluded their use. Instead, data were normalized to the amount of total RNA (0.8 µg) used in cDNA synthesis reactions (Medeiros et al., 2010; Mager et al., 2012). For whole tissues, fold changes are reported relative to whole brain expression (brain expression=1), while brain segment fold changes are reported relative to olfactory bulb expression (olfactory bulb expression=1).

Plasma 5-HT time course upon SERT inhibition with FLX

Toadfish (*N*=7 fish per treatment) were anesthetized in 1 g l⁻¹ MS-222 and surgically implanted with caudal vein and/or caudal arterial catheters (Intramedic PE 50 tubing; Becton Dickinson, Franklin Lakes, NJ, USA) filled with heparinized saline (150 mmol l⁻¹ NaCl with 50 IU ml⁻¹ sodium heparin; Sigma-Aldrich) as described previously (Wood et al., 1997; McDonald et al., 2000). Fish were also implanted with intraperitoneal (IP) catheters (Intramedic PE160 tubing, Becton Dickinson) filled with peanut oil as described previously (McDonald and Walsh, 2004; Medeiros et al., 2014) and sealed with putty. Fish were allowed to recover for 36 h before experiments.

After recovery, initial blood samples ($t=0$) were taken. For these and all subsequent blood samples, a 200 μl pre-sample was first taken to flush the catheter, a 100 μl blood sample was collected, the pre-sample was re-injected along with 100 μl of saline to replace lost blood volume, and the catheter was refilled with heparinized saline. Plasma was separated from red blood cells by centrifugation (10 min at 16,100 g), and 60–70 μl plasma was flash-frozen and stored at -80°C for later analysis of 5-HT. After the initial blood samples, fish were injected with 0, 1, 10 or 50 $\mu\text{g g}^{-1}$ FLX (as fluoxetine hydrochloride; Toronto Research Chemicals, Toronto, Canada) in warmed coconut oil (5 $\mu\text{l g}^{-1}$; Sigma-Aldrich) via IP catheter. IP catheters were then filled with peanut oil to flush the doses into the fish. Blood samples were taken at $t=5$ min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h and 24 h after treatment as described above. Plasma samples were later analysed for 5-HT concentrations using ELISA (ALPCO, Salem, NH, USA).

Statistics

Statistics were performed in Prism (version 7; GraphPad, La Jolla, CA, USA). Data were tested for normality using the Shapiro–Wilk normality test. Non-normal data were log-transformed to meet normality assumptions. Student's t -test was used to compare mean 5-HT uptake between water-injected and cRNA-injected oocytes. A Hill model was fitted to oocyte concentration series uptake data to determine K_m and V_{max} (maximum rate in Michaelis–Menten kinetics) values. Uptake dependence on Na^+ and Cl^- and SERT mRNA distribution in whole tissues was analysed by one-way ANOVA with Tukey's multiple comparisons test. SERT expression data for brain segments could not be transformed to normality and were tested for significant differences using the Kruskal–Wallis test with Dunn's multiple comparisons test. Repeated measures two-way ANOVA with Dunnett's multiple comparisons test was used to analyse plasma 5-HT time course data. Statistically significant differences were defined as $P<0.05$. Means are presented ± 1 s.e.m.

RESULTS

The full-length toadfish SERT protein-coding sequence (GenBank accession number ALK82492.1) is 2022 base pairs long and encodes a protein of 673 amino acids with 12 predicted transmembrane helices (Fig. 1). Translated toadfish SERT shows the highest sequence similarity (90%) with SERT of the teleost *Larimichthys crocea*, diverging at only 65 amino acid residues (Fig. 2). Toadfish SERT is slightly less similar to zebrafish SERT (80%), diverging at 136 residues (Fig. 2), and demonstrates 81–88%

similarity with other examined teleost SERTs, with the exception of zebrafish SERTB (60% similarity). In comparison, toadfish SERT shows less similarity to mammalian SERTs (65–66%), differing from human SERT at 229 residues and from rat SERT at 235 residues (Fig. 2). In phylogenetic analysis, toadfish SERT grouped with SERTs of other teleosts, with the exception of zebrafish SERTB (Fig. 3). Invertebrate SERTs were the most ancestral groupings, mammalian SERTs were more derived, and human and non-human primate SERTs were the most derived groupings (Fig. 3).

At a single 5-HT concentration (2 $\mu\text{mol l}^{-1}$), toadfish SERT cRNA-injected oocytes demonstrated 2.6-fold greater total 5-HT uptake than water-injected controls (Fig. 4A). Analysis of uptake rates over a range of 5-HT concentrations by fitting a specific binding equation with Hill slope ($R^2=0.962$) revealed a K_m for toadfish SERT of $2.08\pm 0.45 \mu\text{mol l}^{-1}$ [95% confidence interval (CI): 0.84–3.33 $\mu\text{mol l}^{-1}$] and a V_{max} of $0.0095\pm 0.0009 \text{ pmol 5-HT min}^{-1}$ (95% CI: 0.0070–0.0121 $\text{pmol 5-HT min}^{-1}$) with a Hill coefficient of 3.19 ± 1.41 (95% CI: -0.73 –7.11; Fig. 4B). Uptake of 5-HT by toadfish SERT was both Na^+ and Cl^- dependent, as shown by a complete lack of uptake in Na^+ - and Cl^- -free incubation media compared with normal medium containing both Na^+ and Cl^- (Fig. 4C).

SERT mRNA expression was detected in all tissues examined. SERT mRNA expression in whole blood was negligible; expression was below the detection threshold in five of eight samples, and samples that did yield Ct values had approximately 14,000-fold lower expression than that in the brain (data not shown). Within the brain, SERT mRNA expression in the cerebellum was approximately 400-fold greater than in the olfactory bulb, and was significantly greater than in all other segments but the diencephalon (Fig. 5A). Brain SERT mRNA expression was lowest in the telencephalon (Fig. 5A). Heart expression was significantly greater than in eight of the other 13 tissues examined, approximately 85- to 149-fold greater than in the tissues with the lowest mean expression – the esophagus, kidney and spleen – and approximately 30-fold higher than in the brain (Fig. 5B). SERT mRNA expression in the liver and stomach was also significantly (approximately 12- to 24-fold) greater than in the esophagus, kidney and spleen, but was not significantly greater than in the brain (Fig. 5B).

Treatment of toadfish with vehicle control (0 $\mu\text{g g}^{-1}$ FLX) and 1 $\mu\text{g g}^{-1}$ FLX via IP injection did not result in significant changes in plasma 5-HT concentrations at any measured time point compared with pre-injection levels (Fig. 6A,B), whereas treatment with 10 $\mu\text{g g}^{-1}$ FLX significantly increased plasma 5-HT 1.6- to 1.7-fold

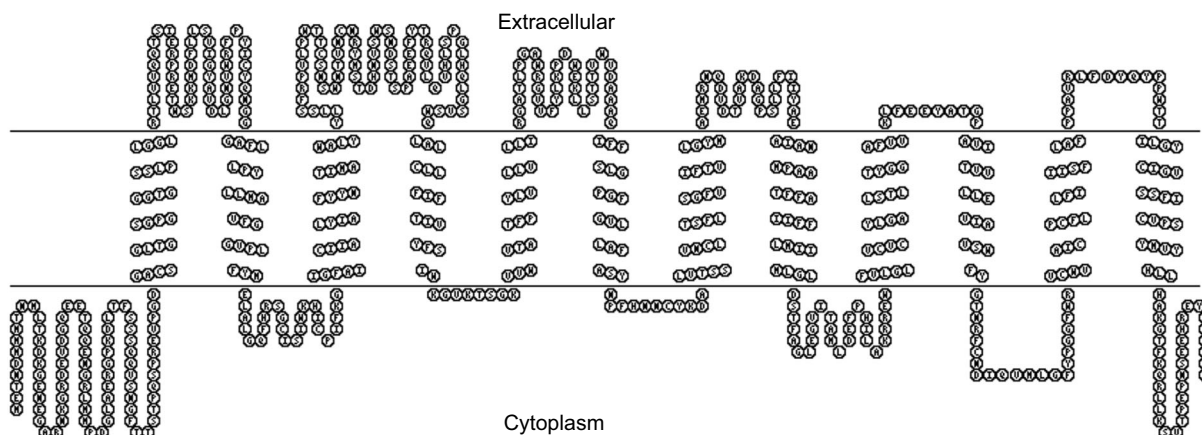


Fig. 1. Predicted secondary structure and membrane organization of the toadfish SERT. Figure generated using MEMSAT2 software.

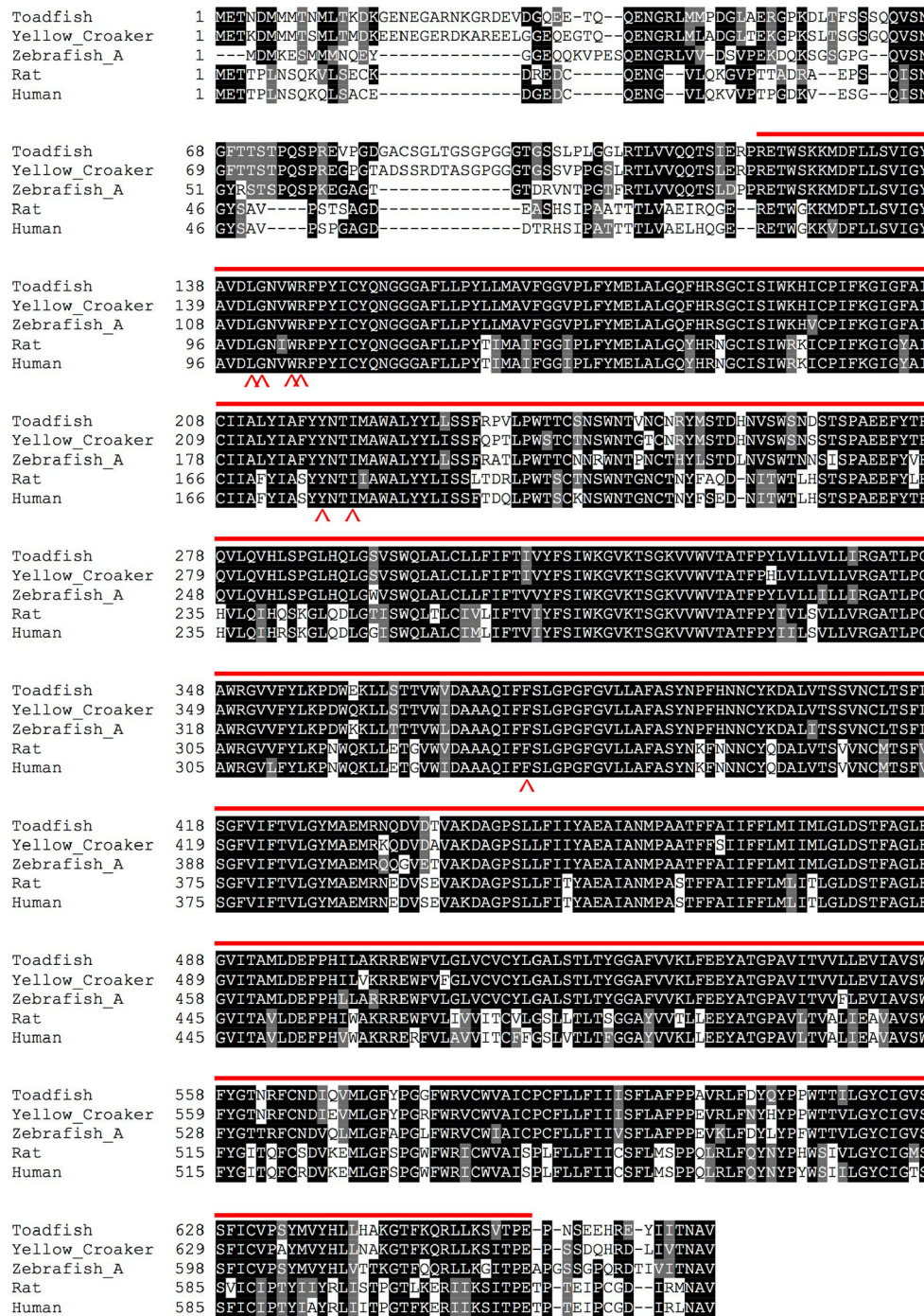


Fig. 2. MUSCLE multiple alignment of amino acid sequences. Toadfish (*Opsanus beta*), ALK82492.1; yellow croaker (*Larimichthys crocea*), KKF08838.1; zebrafish (*Danio rerio*) isoform A, AAI63777.1; rat (*Rattus norvegicus*), NP_037166.2; human (*Homo sapiens*), AAW80933.1. Figure generated using BoxShade after MUSCLE alignment in UGene software. Red overlay denotes predicted solute binding domain (Marchler-Bauer et al., 2017). Red carets (^) denote residues likely to be important for SSRI binding (Zhou et al., 2009).

from 30 min to 2 h after injection (Fig. 6C). In fish treated with the highest dose, 50 $\mu\text{g g}^{-1}$ FLX, plasma 5-HT concentrations were significantly (1.7- to 2.0-fold) elevated from 15 min to 2 h after injection, showing an initial increase, a peak and plateau at 30 min to 1 h after injection, and a subsequent decrease toward pre-injection concentrations (Fig. 6D). There was no overall effect of dose, but there was a significant interaction between time and dose factors ($P < 0.0001$).

DISCUSSION

The 12-transmembrane-helix structure of toadfish SERT, as predicted based on the translated cDNA sequence, is consistent with the surmised structures of other SERTs and with the family of

neurotransmitter transporters in general (Ramamoorthy et al., 1993; Chang et al., 1996; Chen et al., 1998b; reviewed by Nelson, 1998). The high degree of similarity among toadfish SERT and other SERTs suggests considerable conservation of function among species and supports the utility of using teleost model organisms to study SERTs in a broader context – especially because the greatest similarity was retained within the predicted solute-binding domain (Marchler-Bauer et al., 2017). The similarity rises from 90% overall to 95% within this domain for toadfish and *L. crocea*; from 80% overall to 92% within the domain for toadfish and zebrafish SERTA; and from 65–66% overall to 77% within the domain for toadfish and mammalian SERTs. Furthermore, toadfish and human SERTs are identical at putative human Na⁺ and 5-HT binding sites

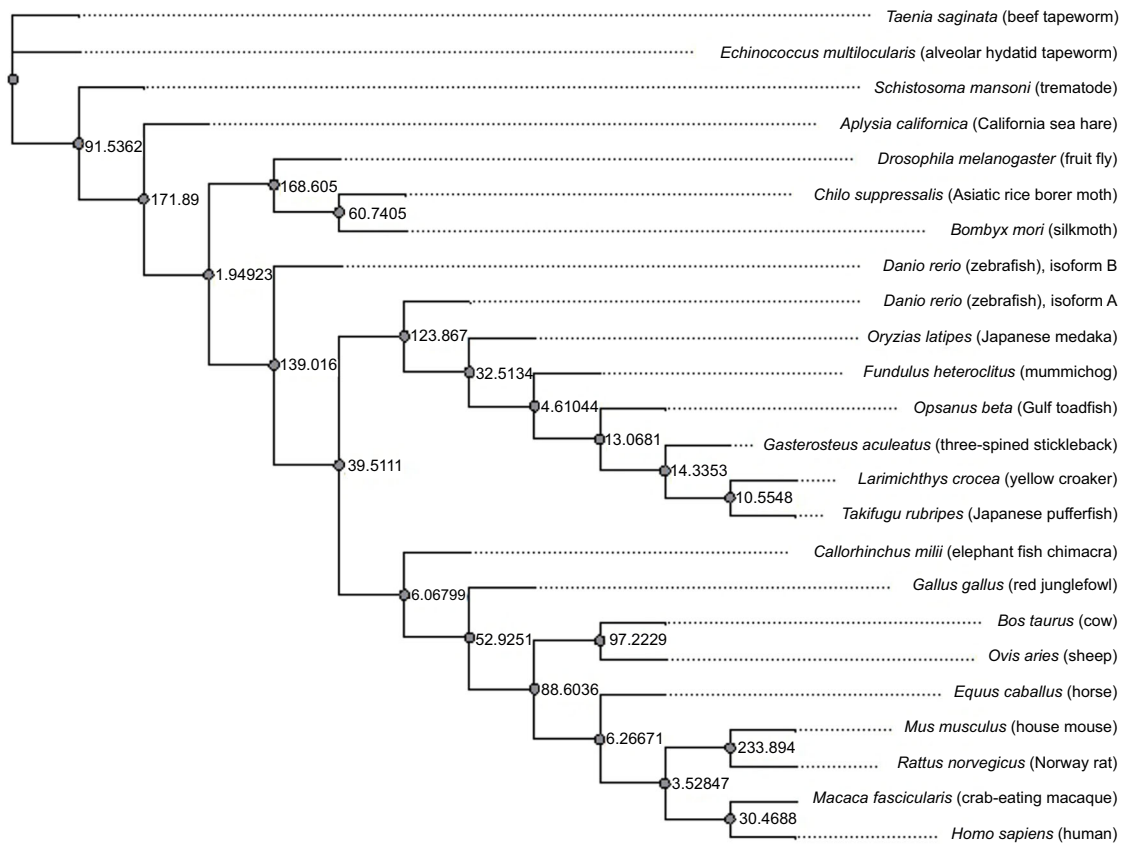


Fig. 3. Phylogenetic analysis of amino acid sequences. Genbank/RefSeq/Ensembl accession numbers, from top: OCK35329.1, CDS36751.1, ABK51389.1, NP_001191502.1, NP_523846.2, AKL78871.1, NP_001037436.1, AAI63371.1, AAI63777.1, XP_004075963.2, JAR86319.1, ALK82492.1, ENSGACP00000008195.1, KKF08838.1, XP_003968295.2, AFO94871.1, AAS79016.1, AAD26262.1, NP_001009446.1, NP_001075293.1, AAB67172.1, NP_037166.2, NP_001274253.1, AAW80933.1. Analysis parameters: method, PhyML maximum likelihood; substitution model, LG; branch support, aLRT method.

(Yamashita et al., 2005; Beuming et al., 2006; Singh et al., 2007, 2008; Zhou et al., 2007; Celik et al., 2008; Shi et al., 2008; Quick et al., 2009; Marchler-Bauer et al., 2017). Binding site conservation also underscores the potential for SSRI pollutants in the environment to affect non-target species like fish, as residues deemed critical for SSRI docking are conserved between human and toadfish SERT (Zhou et al., 2009).

Consistent with the sequence similarities, confirmation of Na^+ and Cl^- dependence in toadfish SERT is in agreement with known mechanisms of action of SERTs and of neurotransmitter transporters more broadly (Amara and Kuhar, 1993; Nelson, 1998). SERTs utilize the movement of Na^+ and Cl^- into the cell – along with outward movement of K^+ or H^+ – to drive uptake of the cationic form of 5-HT (reviewed in Rudnick, 2002). In human SERT, the traditionally proposed stoichiometry (1:1:1:1 $\text{Na}^+:\text{Cl}^-:\text{K}^+:5\text{-HT}^+$) indicates that this process is electroneutral (Rudnick and Nelson, 1978; Nelson and Rudnick, 1979; reviewed in Rudnick, 2002); however, SERT still facilitates inward currents due to leak currents as well as ion fluxes that appear to be associated with inward-facing and/or channel-like conformations of the transporter (Mager et al., 1994; Galli et al., 1997; Adams and DeFelice, 2003; Schicker et al., 2012; reviewed in DeFelice, 2016), and SERT may in fact exhibit variable stoichiometry (Mager et al., 1994). Interaction with the SNARE protein syntaxin 1A ceases leak and uncoupled ion currents in mammalian SERT (Quick, 2003), indicating that SERT-associated currents can be dynamically regulated *in vivo*. Interestingly, FLX also inhibits SERT-associated currents (Mager et al., 1994), raising the possibility

that fluoxetine exposure could alter important ion-dependent processes such as osmoregulation and acid–base balance in organisms like teleosts.

The differences between mammalian and teleost SERT sequences confer, in part, a lower affinity for 5-HT (as indicated by a higher K_m) in teleost SERTs. As calculated from the Hill model, the toadfish SERT K_m ($2.08 \pm 0.45 \mu\text{mol l}^{-1}$) is similar to reported K_m estimates for zebrafish SERTA ($4.20 \mu\text{mol l}^{-1}$ and $2.13 \mu\text{mol l}^{-1}$; Wang et al., 2006; Severinsen et al., 2008), while mammalian K_m estimates range from 0.28 to $0.70 \mu\text{mol l}^{-1}$ (Ramamoorthy et al., 1993; Chang et al., 1996; Owens et al., 1997; Chen et al., 1998b; Mortensen et al., 1999). Such differences in affinity may reflect differences in local internal 5-HT concentrations between these organismal groups (reviewed by McDonald, 2017), and the difference of three orders of magnitude between the toadfish SERT K_m value ($\mu\text{mol l}^{-1}$) and actual circulating concentrations (nmol l^{-1}) are probably indicative of the levels of 5-HT in the immediate vicinity of the transporters (e.g. the synapse or proximal to 5-HT-producing tissues) rather than in the general circulation (see McDonald, 2017). The Hill model fitted our transport data better than the Michaelis–Menten model that has been used for other mammal and fish SERTs (Chang et al., 1996; Mortensen et al., 1999; Wang et al., 2006; Severinsen et al., 2008; Fontana et al., 2009). However, the very broad confidence interval around the Hill coefficient indicates that this parameter does not differ significantly from 1, suggesting the zero cooperativity that is indicative of Michaelis–Menten kinetics and supporting our hypothesis of similar transport kinetics across different organisms.

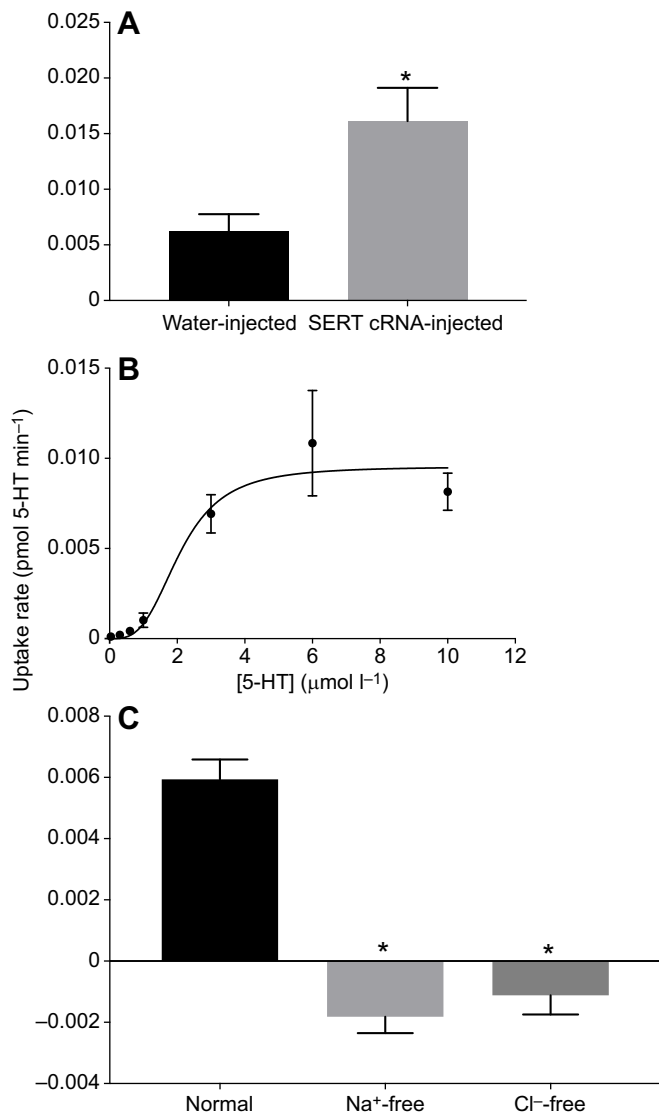


Fig. 4. SERT-mediated 5-HT uptake in *Xenopus laevis* oocytes. (A) 5-HT uptake in cRNA-injected oocytes versus those injected with water (controls). The asterisk (*) denotes a statistically significant difference [$P < 0.05$, Student's t -test, $N = 7$ (cRNA-injected) or $N = 8$ (controls)]. (B) SERT 5-HT transport kinetics. Hill plot of 5-HT uptake rate in cRNA-injected oocytes (non-specific binding subtracted) over a series of 5-HT concentrations ($N = 3$ replicates of 2–6 pooled oocytes per concentration). (C) SERT-mediated 5-HT uptake in the presence of Na⁺ and Cl⁻ ('normal') and in Na⁺-free and Cl⁻-free media. Asterisks (*) denote a statistically significant difference ($P < 0.01$, one-way ANOVA with Tukey's multiple comparisons test, $N = 3$ replicates of 2–6 pooled oocytes per medium type).

The discovery of SERT transcript throughout the toadfish brain is consistent with the widespread expression noted in previous studies on zebrafish and goldfish (Norton et al., 2008; Mennigen et al., 2010b). However, the markedly elevated SERT mRNA expression in the toadfish cerebellum relative to other brain regions is surprising when compared with other fish and mammals. While the goldfish cerebellum contains SERT transcript, levels do not appear higher than other brain regions using semi-quantitative Northern blot (Mennigen et al., 2010a,b), and no SERT transcript has been found in the zebrafish cerebellum using immunohistochemistry (Kaslin and Panula, 2001; Norton et al., 2008). Within mammals, similar discrepancies have been noted. For

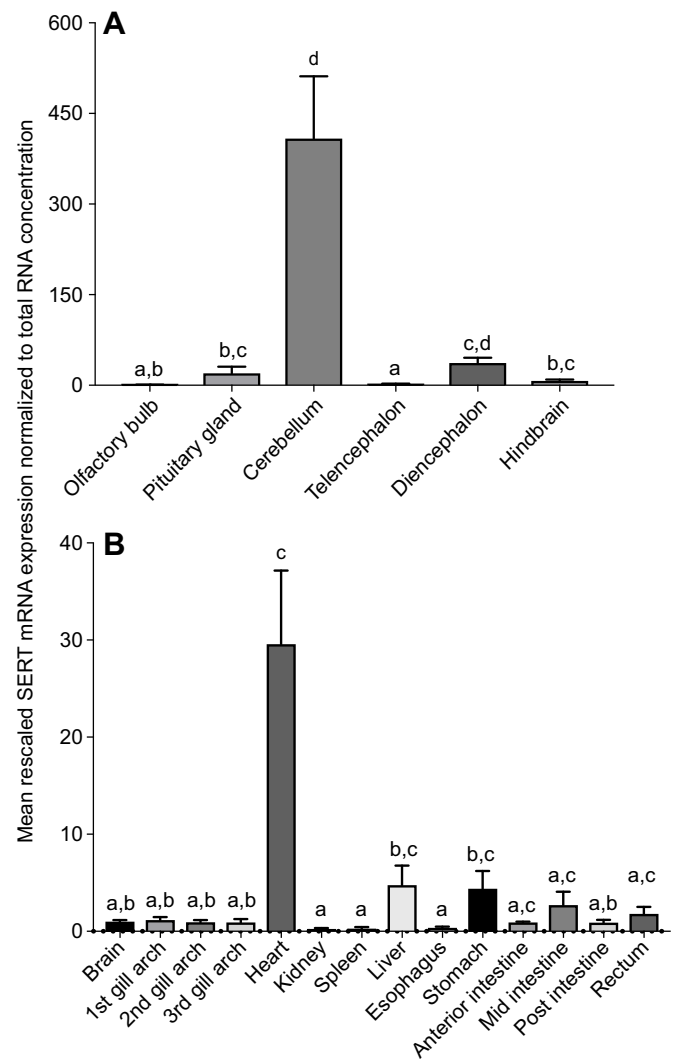


Fig. 5. Mean SERT mRNA expression in toadfish brain segments and a suite of toadfish tissues. (A) Toadfish brain segments. (B) Other toadfish tissues. Expression is normalized to the total RNA concentration used in cDNA synthesis and rescaled relative to expression in the (A) olfactory bulb or (B) brain. Bars not sharing a lowercase letter are significantly different from each other as determined by (A) one-way ANOVA and Tukey's multiple comparisons test [$N = 5$, except for telencephalon and hindbrain ($N = 6$)] or (B) Kruskal–Wallis test and Dunn's multiple comparisons test [$N = 8$ except for heart ($N = 7$) and brain ($N = 16$)]. Brain segments are identified as described in Medeiros et al. (2010).

example, in the human cerebellum, SERT protein is only detectable at very low levels (Kish et al., 2005), and 5-HT uptake sites are undetectable in the mouse cerebellum (Bengel et al., 1997). In contrast, rat cerebellar tissue has been shown to take up 5-HT (Beas-Zarate et al., 1984), and perfusion with the SERT inhibitor FLX increases local 5-HT levels (Mendlin et al., 1996), perhaps suggesting an enhanced role of SERT in the cerebellum of rats compared with humans and mice. Nonetheless, the exceptionally high SERT mRNA expression in the toadfish cerebellum appears to be unprecedented, and the potential implications are unclear. In mammals, the cerebellum is known to be involved in motor control, social interaction, language learning, auditory processing and the control of respiratory responses during progressive hypoxia (Xu et al., 1995; Broussard, 2013; McLachlan and Wilson, 2017). While specific roles of 5-HT signaling within the cerebellum remain

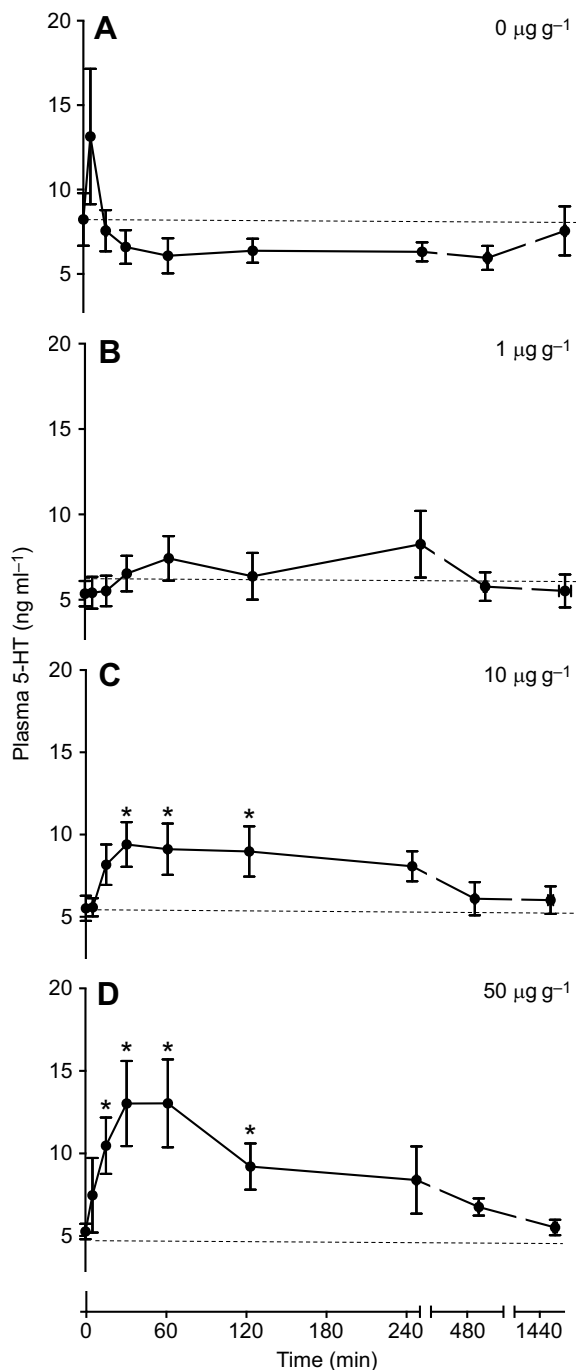


Fig. 6. Plasma 5-HT concentrations over time after SERT inhibition by fluoxetine. Fluoxetine (FLX) concentrations: (A) $0 \mu\text{g g}^{-1}$; (B) $1 \mu\text{g g}^{-1}$; (C) $10 \mu\text{g g}^{-1}$; (D) $50 \mu\text{g g}^{-1}$. Asterisks (*) denote a statistically significant difference from time $t=0$ ($P<0.05$; two-way repeated measures ANOVA with Dunnett's multiple comparisons test; $N=7$).

uncertain, SERT-mediated control of 5-HT in the toadfish cerebellum could possibly contribute to any of these multiple functions. Toadfish engage in significant vocal communication with conspecifics (reviewed in Bass and McKibben, 2003), are highly aggressive (Sloman et al., 2005; McDonald et al., 2011), and are remarkably tolerant of a variety of environmental stressors including hypoxia (Hall, 1929; Ultsch et al., 1981) and ammonia (Wang and Walsh, 2000). It is therefore conceivable that SERT,

perhaps associated with a high density of serotonergic neurons within this region, could somehow be involved in modulating these specialized adaptations. However, evidence is thus far lacking, and such speculation necessitates further investigation into the functions of cerebellar 5-HT in this species.

In addition to its existence in the brain, the presence of SERT transcript in all 13 peripheral tissues examined highlights the ubiquity of the transporter and is consistent with the widespread peripheral expression reported in mammals (Lesch et al., 1993b; Chang et al., 1996; Chen et al., 1998a; Mortensen et al., 1999; Ni et al., 2004; Pavone et al., 2008, 2009; Yokoyama et al., 2013; reviewed in Ni and Watts, 2006) and in another teleost, the goldfish (Mennigen et al., 2010b). Interestingly, the high SERT mRNA expression in the toadfish heart compared with other tissues appears to be consistent with its expression in goldfish determined using northern blot analysis (Mennigen et al., 2010b), although to our knowledge, SERT mRNA expression in the heart tissue of any other species has not been examined. However, considerable levels of 5-HT have been found in the ventricle of the European conger (Piomelli and Tota, 1983) [although not in the ventricles of rainbow trout or zebrafish (Caamaño-Tubío et al., 2007; Stoyek et al., 2017)], and two separate populations of 5-HT-immunoreactive cells have been identified in the zebrafish atrium (Stoyek et al., 2017). These atrial cells appear to store 5-HT but lack the 5-HT synthesis enzyme, tryptophan hydroxylase (Stoyek et al., 2017). Combined with our results, this may suggest that 5-HT sequestration by the heart atrium is not due to production but may be SERT mediated.

Although definitive reasons are absent, it is possible that high SERT mRNA expression in the fish heart, especially in the epithelia, could be reflective of the heart's location within the circulatory system – immediately before the gill (Laurent, 1984). Within the circulation, 5-HT causes constriction of branchial blood vessels and reduces gas exchange (Fritsche et al., 1992); one could hypothesize that removal of 5-HT from the circulation could control what may be detrimental if left unchecked. Thus, SERT within the heart (if indeed mRNA expression translates to protein function) as well as SERT in the gill itself could provide a mechanism by which 5-HT could be sequestered from the bloodstream before reaching 5-HT-sensitive areas in the gill such as the efferent filamentary artery sphincter – a major site of blood flow control in the gill (Nilsson and Sundin, 1998; Sundin and Nilsson, 2000; Pelster and Schwerte, 2012). A tissue-based sequestration mechanism would be particularly important given our finding that toadfish blood does not contain appreciable levels of SERT transcript – as well as findings from other studies that neither fish thrombocytes (the evolutionary precursors to mammalian platelets) nor other blood cells appear to play a role in 5-HT storage and sequestration as a mechanism to control vascular resistance, as is observed for mammalian platelets (Fänge, 1992; Caamaño-Tubío et al., 2007; Mercado and Kilic, 2010). As systemic inhibition of SERT with FLX attenuates cardiovascular aspects of the toadfish hypoxia response (Panlilio et al., 2016) in addition to the overall metabolic response to hypoxia in this fish (Amador et al., 2018), it is possible that cardiac SERT could play a specific role in the hypoxia response in hypoxia-tolerant species such as toadfish (Hall, 1929; Ultsch et al., 1981; McDonald et al., 2010) and goldfish (reviewed in Bickler and Buck, 2007).

In addition to the heart, toadfish SERT mRNA expression was also elevated in the liver compared with several other tissues. SERT transcript has likewise been found in the liver of goldfish, although levels relative to other tissues are unclear (Mennigen et al., 2010b).

It is possible that elevated SERT in the toadfish liver could be linked to a role in 5-HT synthesis. Tryptophan hydroxylase is present at high levels in the liver of several teleosts and in the stomach of one species examined (Nagai et al., 1997). Therefore, concurrent high levels of SERT expression and 5-HT production in the liver could enable the regulation of synthesized 5-HT and its movement to other tissues. The liver may also play a specific role in 5-HT sequestration from the bloodstream, as speculated for the heart, as it is a major site of 5-HT metabolism in both mammals (reviewed in Ruddell et al., 2008) and rainbow trout (Caamaño-Tubío et al., 2007). Interestingly, in mammals, peripheral 5-HT is synthesized mainly in the enterochromaffin cells of the intestine (not the liver), and SERT, located in close proximity, transports and regulates this newly synthesized 5-HT (Anderson and Campbell, 1988; Kiliaan et al., 1989; Olsson et al., 2008; reviewed in Mawe and Hoffman, 2013). In contrast, many teleosts lack intestinal enterochromaffin cells, and the intestine as a whole does not have measurable tryptophan hydroxylase activity (Nagai et al., 1997). That being said, the highest levels of 5-HT in the rainbow trout periphery are still associated with the intestine but are in nerve fibers (Caamaño-Tubío et al., 2007). Thus, in contrast to the mammalian intestine, the teleost intestine might obtain its 5-HT via uptake rather than production, similar to the heart atrial cells (Caamaño-Tubío et al., 2007; Stoyek et al., 2017).

Systemic inhibition of SERT via FLX treatment enabled a broader assessment of the potential contribution of tissues in the control of circulating 5-HT. Previous findings of increased plasma 5-HT concentrations after FLX treatment in toadfish (Morando et al., 2009; McDonald et al., 2011) suggests the presence of SERT-mediated 5-HT clearance from the blood. As there is not a significant circulating cellular 5-HT storage pool in teleosts (Caamaño-Tubío et al., 2007), we suspected that, under normal conditions, tissues throughout the periphery were responsible for the uptake of SERT and thus for maintenance of baseline levels of circulating 5-HT in toadfish. Indeed, the widespread tissue distribution of toadfish SERT found in the current study further strengthened the hypothesis that there would be initial increases in plasma 5-HT after FLX treatment. Our results generally support this hypothesis, as no increase was observed at the lowest dose ($1 \mu\text{g g}^{-1}$) of FLX (comparable to the upper limit of typical therapeutic doses in humans; see McDonald, 2017), a moderate increase was evident at $10 \mu\text{g g}^{-1}$ FLX, and a dramatic and rapid increase over several time points was measured at $50 \mu\text{g g}^{-1}$ FLX. Such results indicate a progressive blockade of systemic SERT and suggest that SERT in tissues does play a substantial role in modulating peripheral 5-HT dynamics.

That plasma 5-HT concentrations were not elevated 24 h after administration of even the highest dose ($50 \mu\text{g g}^{-1}$) of FLX is in contrast to previous studies in toadfish in which the same and lower doses of FLX, also administered intraperitoneally in coconut oil, caused significant increases in circulating 5-HT measured 24–40 h after implantation (Morando et al., 2009; McDonald et al., 2011). However, the implants in previous studies were solidified on ice immediately after injection to allow slower release of the compound over time. Warmed coconut oil was used in the present study and was not solidified after implantation, probably contributing to faster absorption and subsequent elimination of implanted FLX and therefore faster return of SERT function. Faster absorption would also explain the rapid increase in plasma 5-HT at the highest dose of FLX – a 98% increase within 15 min. Nonetheless, despite differences among studies, the current results clearly demonstrate the importance of systemic SERT in contributing to 5-HT homeostasis in the periphery.

Conclusions

We have shown that the Gulf toadfish SERT demonstrates high levels of conservation with other teleost SERTs with regard to sequence and function, and that the transporter is ubiquitous within the brain and peripheral tissues of this fish but is absent or present at negligible levels in the blood. Furthermore, the importance of peripheral SERT in contributing to 5-HT homeostasis is evident based on dose- and time-dependent effects of SERT inhibition on circulating 5-HT. The characterization of toadfish SERT presented here thus provides valuable context within which to interpret existing and future studies on 5-HT signaling in the Gulf toadfish and other teleosts. Ultimately, the degree of conservation among teleost and mammalian SERTs underscores the utility of teleost fish as model organisms in 5-HT research – and also emphasizes the potential for SERT-inhibiting pollutants to affect these non-target species.

Acknowledgements

The authors would like to acknowledge Dr Lea R. Medeiros for contributing brain segment cDNA; Dr Christophe M. R. LeMoine and Dr Kevin L. Schauer for their help mining the toadfish transcriptome; Dr Edward M. Mager and Dr Ilan M. Ruhr for their guidance in primer design and qPCR techniques; Dr Gerhard P. Dahl for his generous donations of oocytes and advice on oocyte assays; and Dr Lynne A. Fieber for providing advice and necessary equipment for oocyte maintenance and injection.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.H.B.A., M.D.M.; Methodology: M.H.B.A., M.D.M.; Formal analysis: M.H.B.A.; Investigation: M.H.B.A.; Resources: M.D.M.; Writing - original draft: M.H.B.A.; Writing - review & editing: M.H.B.A., M.D.M.; Visualization: M.H.B.A., M.D.M.; Supervision: M.D.M.; Project administration: M.H.B.A., M.D.M.; Funding acquisition: M.H.B.A., M.D.M.

Funding

This research was supported by a University of Miami Graduate Fellowship to M.H.B.A. and a University of Miami Provost Research Award to M.D.M.

Data availability

The toadfish SERT mRNA sequence has been deposited in GenBank with accession no. KR866115.1 (www.ncbi.nlm.nih.gov/nuccore/941508005). The corresponding amino acid translation has accession no. ALK82492.1 (www.ncbi.nlm.nih.gov/protein/941508006).

Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.170928.supplemental>

References

- Adams, S. V. and DeFelice, L. J. (2003). Ionic currents in the human serotonin transporter reveal inconsistencies in the alternating access hypothesis. *Biophys. J.* **85**, 1548–1559.
- Amador, M. H. B., Schauer, K. L. and McDonald, M. D. (2018). Does fluoxetine exposure affect hypoxia tolerance in the Gulf toadfish, *Opsanus beta*? *Aquat. Toxicol.* In Press.
- Amara, S. G. and Kuhar, M. J. (1993). Neurotransmitter transporters: recent progress. *Annu. Rev. Neurosci.* **16**, 73–93.
- Anderson, C. and Campbell, G. (1988). Immunohistochemical study of 5-HT-containing neurons in the teleost intestine: relationship to the presence of enterochromaffin cells. *Cell Tissue Res.* **254**, 553–559.
- Barkan, T., Gurwitz, D., Levy, G., Weizman, A. and Rehavi, M. (2004). Biochemical and pharmacological characterization of the serotonin transporter in human peripheral blood lymphocytes. *Eur. Neuropsychopharmacol.* **14**, 237–243.
- Barnes, K. K., Kolpin, D. W., Furlong, E. T., Zaugg, S. D., Meyer, M. T. and Barber, L. B. (2008). A national reconnaissance of pharmaceuticals and other organic wastewater contaminants in the United States – I. groundwater. *Sci. Total Environ.* **402**, 192–200.
- Bass, A. H. and McKibben, J. R. (2003). Neural mechanisms and behaviors for acoustic communication in teleost fish. *Prog. Neurobiol.* **69**, 1–26.

- Beas-Zarate, C., Sandoval, M. E. and Feria-Velasco, A. (1984). Serotonin uptake and release from rat cerebellum *in vitro*. *J. Neurosci. Res.* **12**, 129-136.
- Bengel, D., Jöhren, O., Andrews, A. M., Heils, A., Mössner, R., Sanvitto, G. L., Saavedra, J. M., Lesch, K.-P. and Murphy, D. L. (1997). Cellular localization and expression of the serotonin transporter in mouse brain. *Brain Res.* **778**, 338-345.
- Berger, M., Gray, J. A. and Roth, B. L. (2009). The expanded biology of serotonin. *Annu. Rev. Med.* **60**, 355-366.
- Bernier, N. J. and Perry, S. F. (1996). Control of catecholamine and serotonin release from the chromaffin tissue of the Atlantic hagfish. *J. Exp. Biol.* **199**, 2485-2497.
- Beuming, T., Shi, L., Javitch, J. A. and Weinstein, H. (2006). A comprehensive structure-based alignment of prokaryotic and eukaryotic neurotransmitter/Na⁺ symporters (NSS) aids in the use of the LeuT structure to probe NSS structure and function. *Mol. Pharmacol.* **70**, 1630-1642.
- Bickler, P. E. and Buck, L. T. (2007). Hypoxia tolerance in reptiles, amphibians, and fishes: life with variable oxygen availability. *Annu. Rev. Physiol.* **69**, 145-170.
- Blakely, R. D., Berson, H. E., Freneau, R. T., Caron, M. G., Peek, M. M., Prince, H. K. and Bradley, C. C. (1991). Cloning and expression of a functional serotonin transporter from rat brain. *Nature* **354**, 66-70.
- Brooks, B. W., Chambliss, C. K., Stanley, J. K., Ramirez, A., Banks, K. E., Johnson, R. D. and Lewis, R. J. (2005). Determination of select antidepressants in fish from an effluent-dominated stream. *Environ. Toxicol. Chem.* **24**, 464-469.
- Brooks, B. W., Foran, C. M., Richards, S. M., Weston, J., Turner, P. K., Stanley, J. K., Solomon, K. R., Slattery, M. and La Point, T. W. (2003). Aquatic ecotoxicology of fluoxetine. *Toxicol. Lett.* **142**, 169-183.
- Broussard, D. (2013). *Cerebellum: Learning Movement, Language, and Social Skills*. Ames, IA: Wiley.
- Buddington, R. K. and Krogh, Å. (2004). Hormonal regulation of the fish gastrointestinal tract. *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* **139**, 261-271.
- Caamaño-Tubío, R. I., Pérez, J., Ferreira, S. and Aldegunde, M. (2007). Peripheral serotonin dynamics in the rainbow trout (*Oncorhynchus mykiss*). *Comp. Biochem. Physiol. C-Toxicol. Pharmacol.* **145**, 245-255.
- Celik, L., Schiödt, B. and Tajkhorshid, E. (2008). Substrate binding and formation of an occluded state in the leucine transporter. *Biophys. J.* **94**, 1600-1612.
- Chang, A. S., Chang, S. M., Starnes, D. M., Schroeter, S., Bauman, A. L. and Blakely, R. D. (1996). Cloning and expression of the mouse serotonin transporter. *Mol. Brain Res.* **43**, 185-192.
- Chen, J.-G., Liu-Chen, S. and Rudnick, G. (1998a). Determination of external loop topology in the serotonin transporter by site-directed chemical labeling. *J. Biol. Chem.* **273**, 12675-12681.
- Chen, J. X., Pan, H., Rothman, T. P., Wade, P. R. and Gershon, M. D. (1998b). Guinea pig 5-HT transporter: cloning, expression, distribution, and function in intestinal sensory reception. *Am. J. Physiol.* **275**, G433-G448.
- DeFelice, L. J. (2016). A current view of serotonin transporters. *F1000Res.* **5**, 1-7.
- Duncan, G. E., Little, K. Y., Kirkman, J. A., Kaldas, R. S., Stumpf, W. E. and Brees, G. R. (1992). Autoradiographic characterization of [³H]mipramine and [³H]citalopram binding in rat and human brain: species differences and relationships to serotonin innervation patterns. *Brain Res.* **591**, 181-197.
- El-Merabhi, R., Löffler, M., Mayer, A. and Sumara, G. (2015). The roles of peripheral serotonin in metabolic homeostasis. *FEBS Lett.* **589**, 1728-1734.
- Fänge, R. (1992). Fish blood cells. In *Fish Physiology* (ed. W. Hoar, D. Randall and A. P. Farrell), pp. 1-54. Amsterdam: Elsevier.
- Ferriere, F., Khan, N. A., Meyniel, J.-P. and Deschaux, P. (1999). Characterisation of serotonin transport mechanisms in rainbow trout peripheral blood lymphocytes: role in PHA-induced lymphoproliferation. *Dev. Comp. Immunol.* **23**, 37-50.
- Fontana, A. C. K., Sonders, M. S., Pereira-Junior, O. S., Knight, M., Javitch, J. A., Rodrigues, V., Amara, S. G. and Mortensen, O. V. (2009). Two allelic isoforms of the serotonin transporter from *Schistosoma mansoni* display electrogenic transport and high selectivity for serotonin. *Eur. J. Pharmacol.* **616**, 48-57.
- Forster, M. E., Forster, A. H. and Davison, W. (1998). Effects of serotonin, adrenaline and other vasoactive drugs on the branchial blood vessels of the Antarctic fish *Pagothenia borchgrevinki*. *Fish Physiol. Biochem.* **19**, 103-109.
- Fritsche, R., Reid, S. G., Thomas, S. and Perry, S. F. (1993). Serotonin-mediated release of catecholamines in the rainbow trout *Oncorhynchus mykiss*. *J. Exp. Biol.* **178**, 191-204.
- Fritsche, R., Thomas, S. and Perry, S. F. (1992). Effects of serotonin on circulation and respiration in the rainbow trout *Oncorhynchus mykiss*. *J. Exp. Biol.* **73**, 59-73.
- Fuller, R. W. and Wong, D. T. (1990). Serotonin uptake and serotonin uptake inhibition. *Ann. N. Y. Acad. Sci.* **600**, 68-80.
- Galli, A., Petersen, C. I., DeBlaquiere, M., Blakely, R. D. and DeFelice, L. J. (1997). *Drosophila* serotonin transporters have voltage-dependent uptake coupled to a serotonin-gated ion channel. *J. Neurosci.* **17**, 3401-3411.
- Hall, F. G. (1929). The influence of varying oxygen tensions upon the rate of oxygen consumption in marine fishes. *Am. J. Physiol.* **88**, 212-218.
- Janvier, J.-J., Peyraud-Waitzenegger, M. and Soulier, P. (1996). Effects of serotonin on the cardio-circulatory system of the European eel (*Anguilla anguilla*) *in vivo*. *J. Comp. Physiol. B.* **166**, 131-137.
- Jones, D. T., Taylor, W. R. and Thornton, J. M. (1994). A model recognition approach to the prediction of all-helical membrane protein structure and topology. *Biochemistry* **33**, 3038-3049.
- Kaslin, J. and Panula, P. (2001). Comparative anatomy of the histaminergic and other aminergic systems in zebrafish (*Danio rerio*). *J. Comp. Neurol.* **440**, 342-377.
- Kiliaan, A. J., Dekker, K. and Groot, J. A. (1989). Serotonergic neurons in the intestine of two teleosts, *Carassius auratus* and *Oreochromis mossambicus*, and the effect of serotonin in transepithelial ion-selectivity and muscle tension. *Neuroscience* **31**, 817-824.
- Kish, S. J., Furukawa, Y., Chang, L.-J., Tong, J., Ginovart, N., Wilson, A., Houle, S. and Meyer, J. H. (2005). Regional distribution of serotonin transporter protein in postmortem human brain: is the cerebellum a SERT-free brain region? *Nucl. Med. Biol.* **32**, 123-128.
- Kolpin, D. W., Furlong, E. T., Meyer, M. T., Thurman, E. M., Zaugg, S. D., Barber, L. B. and Buxton, H. T. (2002). Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: a national reconnaissance. *Environ. Sci. Technol.* **36**, 1202-1211.
- Laurent, P. (1984). Gill internal morphology. In *Fish Physiology* (ed. W. S. Hoar and D. J. Randall), pp. 73-183. Amsterdam: Elsevier.
- Lesch, K. P., Aulakh, C. S., Wolozin, B. L., Tolliver, T. J., Hill, J. L. and Murphy, D. L. (1993a). Regional brain expression of serotonin transporter mRNA and its regulation by reuptake inhibiting antidepressants. *Mol. Brain Res.* **17**, 31-35.
- Lesch, K.-P., Wolozin, B. L., Murphy, D. L. and Reiderer, P. (1993b). Primary structure of the human platelet serotonin uptake site: identity with the brain serotonin transporter. *J. Neurochem.* **60**, 2319-2322.
- Lim, J. E., Porteus, C. S. and Bernier, N. J. (2013). Serotonin directly stimulates cortisol secretion from the interrenals in goldfish. *Gen. Comp. Endocrinol.* **192**, 246-255.
- Linder, A. E., Beggs, K. M., Burnett, R. J. and Watts, S. W. (2009). Body distribution of infused serotonin in rats. *Clin. Exp. Pharmacol. Physiol.* **36**, 599-601.
- Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔCT} method. *Methods* **25**, 402-408.
- Mager, E. M., Medeiros, L. R., Lange, A. P. and McDonald, M. D. (2012). The toadfish serotonin 2A (5-HT_{2A}) receptor: molecular characterization and its potential role in urea excretion. *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* **163**, 319-326.
- Mager, S., Min, C., Henry, D. J., Chavkin, C., Hoffman, B. J., Davidson, N. and Lester, H. A. (1994). Conducting states of a mammalian serotonin transporter. *Neuron* **12**, 845-859.
- Marazziti, D., Rossi, A., Giannaccini, G., Baroni, S., Lucacchini, A. and Cassano, G. B. (1998). Presence and characterization of the serotonin transporter in human resting lymphocytes. *Neuropsychopharmacology* **19**, 154-159.
- Marchler-Bauer, A., Bo, Y., Han, L., He, J., Lanczycki, C. J., Lu, S., Chitsaz, F., Derbyshire, M. K., Geer, R. C., Gonzales, N. R. et al. (2017). CDD/SPARCLE: functional classification of proteins via subfamily domain architectures. *Nucleic Acids Res.* **45**, D200-D203.
- Maurer-Spurej, E. (2005). Circulating serotonin in vertebrates. *Cell. Mol. Life Sci.* **62**, 1881-1889.
- Mawe, G. M. and Hoffman, J. M. (2013). Serotonin signalling in the gut – functions, dysfunctions and therapeutic targets. *Nat. Rev. Gastroenterol. Hepatol.* **10**, 473-486.
- McDonald, M. (2017). An AOP analysis of selective serotonin reuptake inhibitors (SSRIs) for fish. *Comp. Biochem. Physiol. C-Toxicol. Pharmacol.* **197**, 19-31.
- McDonald, M. D., Gilmour, K. M., Walsh, P. J. and Perry, S. F. (2010). Cardiovascular and respiratory reflexes of the gulf toadfish (*Opsanus beta*) during acute hypoxia. *Respir. Physiol. Neurobiol.* **170**, 59-66.
- McDonald, M. D., Gonzalez, A. and Sloman, K. A. (2011). Higher levels of aggression are observed in socially dominant toadfish treated with the selective serotonin reuptake inhibitor, fluoxetine. *Comp. Biochem. Physiol. C-Toxicol. Pharmacol.* **153**, 107-112.
- McDonald, M. D. and Walsh, P. J. (2004). Dogmas and controversies in the handling of nitrogenous wastes: 5-HT₂-like receptors are involved in triggering pulsatile urea excretion in the gulf toadfish, *Opsanus beta*. *J. Exp. Biol.* **207**, 2003-2010.
- McDonald, M. D., Wood, C. M., Wang, Y. and Walsh, P. J. (2000). Differential branchial and renal handling of urea, acetamide and thiourea in the gulf toadfish *Opsanus beta*: evidence for two transporters. *J. Exp. Biol.* **203**, 1027-1037.
- McLachlan, N. M. and Wilson, S. J. (2017). The contribution of brainstem and cerebellar pathways to auditory recognition. *Front. Psychol.* **8**, 265.
- Medeiros, L. R., Cartolano, M. C. and McDonald, M. D. (2014). Crowding stress inhibits serotonin 1A receptor-mediated increases in corticotropin-releasing factor mRNA expression and adrenocorticotropin hormone secretion in the Gulf toadfish. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **184**, 259-271.
- Medeiros, L. R., Mager, E. M., Grosell, M. and McDonald, M. D. (2010). The serotonin subtype 1A receptor regulates cortisol secretion in the Gulf toadfish, *Opsanus beta*. *Gen. Comp. Endocrinol.* **168**, 377-387.

- Medeiros, L. R. and McDonald, M. D. (2012). Elevated cortisol inhibits adrenocorticotrophic hormone- and serotonin-stimulated cortisol secretion from the interrenal cells of the Gulf toadfish (*Opsanus beta*). *Gen. Comp. Endocrinol.* **179**, 414-420.
- Mendlin, A., Martín, F. J., Rueter, L. E. and Jacobs, B. L. (1996). Neuronal release of serotonin in the cerebellum of behaving rats: an *in vivo* microdialysis study. *J. Neurochem.* **67**, 617-622.
- Mennigen, J. A., Lado, W. E., Zamora, J. M., Duarte-Guterman, P., Langlois, V. S., Metcalfe, C. D., Chang, J. P., Moon, T. W. and Trudeau, V. L. (2010b). Waterborne fluoxetine disrupts the reproductive axis in sexually mature male goldfish, *Carassius auratus*. *Aquat. Toxicol.* **100**, 354-364.
- Mennigen, J. A., Sassine, J., Trudeau, V. L. and Moon, T. W. (2010a). Waterborne fluoxetine disrupts feeding and energy metabolism in the goldfish *Carassius auratus*. *Aquat. Toxicol.* **100**, 128-137.
- Mercado, C. P. and Kilic, F. (2010). Molecular mechanisms of SERT in platelets: regulation of plasma serotonin levels. *Rev. Lit. Arts Am.* **10**, 231-241.
- Morando, M. B., Medeiros, L. R. and McDonald, M. D. (2009). Fluoxetine treatment affects nitrogen waste excretion and osmoregulation in a marine teleost fish. *Aquat. Toxicol.* **95**, 164-171.
- Mori, Y. and Ando, M. (1991). Regulation of ion and water transport across the eel intestine: effects of acetylcholine and serotonin. *J. Comp. Physiol. B* **161**, 387-392.
- Mortensen, O. V., Kristensen, A. S., Rudnick, G. and Wiborg, O. (1999). Molecular cloning, expression and characterization of a bovine serotonin transporter. *Brain Res. Mol. Brain Res.* **71**, 120-126.
- Nagai, T., Hamada, M., Kai, N., Tanoue, Y. and Nagayama, F. (1997). Organ distribution of tryptophan hydroxylase activity in several fish. *Fish. Sci.* **63**, 652-653.
- Nelson, N. (1998). The family of Na⁺/Cl⁻ neurotransmitter transporters. *J. Neurochem.* **71**, 1785-1803.
- Nelson, P. J. and Rudnick, G. (1979). Coupling between platelet 5-hydroxytryptamine and potassium transport. *J. Biol. Chem.* **254**, 10084-10089.
- Ni, W., Thompson, J. M., Northcott, C. A., Lookingland, K. and Watts, S. W. (2004). The serotonin transporter is present and functional in peripheral arterial smooth muscle. *J. Cardiovasc. Pharmacol.* **43**, 770-781.
- Ni, W. and Watts, S. W. (2006). 5-Hydroxytryptamine in the cardiovascular system: focus on the serotonin transporter (SERT). *Clin. Exp. Pharmacol. Physiol.* **33**, 575-583.
- Nilsson, S. and Sundin, L. (1998). Gill blood flow control. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* **119**, 137-147.
- Norton, W. H. J., Folchert, A. and Bally-Cuif, L. (2008). Comparative analysis of serotonin receptor (HTR1A/HTR1B families) and transporter (slc6a4a/b) gene expression in the zebrafish brain. *J. Comp. Neurol.* **511**, 521-542.
- Olsson, C., Holmberg, A. and Holmgren, S. (2008). Development of enteric and vagal innervation of the zebrafish (*Danio rerio*) gut. *J. Comp. Neurol.* **508**, 756-770.
- Owens, M. J., Morgan, W. N., Plott, S. J. and Nemeroff, C. B. (1997). Neurotransmitter receptor and transporter binding profile of antidepressants and their metabolites. *J. Pharmacol. Exp. Ther.* **283**, 1305-1322.
- Panlilio, J. M., Marin, S., Lobl, M. B. and McDonald, M. D. (2016). Treatment with the selective serotonin reuptake inhibitor, fluoxetine, attenuates the fish hypoxia response. *Sci. Rep.* **6**, 31148.
- Pavone, L. M., Spina, A., Lo Muto, R., Santoro, D., Mastellone, V. and Avallone, L. (2008). Heart valve cardiomyocytes of mouse embryos express the serotonin transporter SERT. *Biochem. Biophys. Res. Commun.* **377**, 419-422.
- Pavone, L. M., Tafuri, S., Lo Muto, R., Spina, A., Lychkova, A. E., Mastellone, V., Vittoria, A., Avallone, L. and Staiano, N. (2009). Expression of the serotonin transporter SERT in the genital tract of cattle. *Regul. Pept.* **154**, 1-4.
- Pellegrino, D., Acierno, R. and Tota, B. (2003). Control of cardiovascular function in the icefish *Chionodraco hamatus*: involvement of serotonin and nitric oxide. *Comp. Biochem. Physiol. A-Mol. Integr. Physiol.* **134**, 471-480.
- Pelster, B. and Schwerte, T. (2012). The paracrine role of 5-HT in the control of gill blood flow. *Respir. Physiol. Neurobiol.* **184**, 340-346.
- Piomelli, D. and Tota, B. (1983). Different distribution of serotonin in an elasmobranch (*Scyliorhinus stellaris*) and in a teleost (*Conger conger*) fish. *Comp. Biochem. Physiol. C-Pharmacol. Toxicol. Endocrinol.* **74**, 139-142.
- Quick, M. W. (2003). Regulating the conducting states of a mammalian serotonin transporter. *Neuron* **40**, 537-549.
- Quick, M., Winther, A.-M. L., Shi, L., Nissen, P., Weinstein, H. and Javitch, J. A. (2009). Binding of an octylglucoside detergent molecule in the second substrate (S2) site of LeuT establishes an inhibitor-bound conformation. *Proc. Natl. Acad. Sci. USA* **106**, 5563-5568.
- Ramamoorthy, S., Bauman, A. L., Moore, K. R., Han, H., Yang-Feng, T., Chang, A. S., Ganapathy, V. and Blakely, R. D. (1993). Antidepressant- and cocaine-sensitive human serotonin transporter: molecular cloning, expression, and chromosomal localization. *Proc. Natl. Acad. Sci. USA* **90**, 2542-2546.
- Ramirez, A. J., Brain, R. A., Usenko, S., Mottaleb, M. A., O'Donnell, J. G., Stahl, L. L., Wathen, J. B., Snyder, B. D., Pitt, J. L., Perez-Hurtado, P. et al. (2009). Occurrence of pharmaceuticals and personal care products in fish: results of a national pilot study in the United States. *Environ. Toxicol. Chem.* **28**, 2587-2597.
- Ruddell, R. G., Mann, D. A. and Ramm, G. A. (2008). The function of serotonin within the liver. *J. Hepatol.* **48**, 666-675.
- Rudnick, G. (2002). Mechanisms of biogenic amine neurotransmitter transporters. In *Neurotransmitter Transporters: Structure, Function, and Regulation* (ed. M. E. A. Reith), pp. 25-52. Totowa: Humana Press.
- Rudnick, G. and Nelson, P. J. (1978). Platelet 5-hydroxytryptamine transport, an electroneutral mechanism coupled to potassium. *Biochemistry* **17**, 4739-4742.
- Schauer, K. L., Lemoine, C. M. R., Pelin, A., Corradi, N., McDonald, M. D., Warren, W. C. and Grosell, M. (2017). Corrigendum: a proteinaceous organic matrix regulates carbonate mineral production in the marine teleost intestine. *Sci. Rep.* **7**, 46844.
- Schicker, K., Uzelac, Z., Gesmonde, J., Bulling, S., Stockner, T., Freissmuth, M., Boehm, S., Rudnick, G., Sitte, H. H. and Sandtner, W. (2012). Unifying concept of serotonin transporter-associated currents. *J. Biol. Chem.* **287**, 438-445.
- Severinsen, K., Sinning, S., Müller, H. K. and Wiborg, O. (2008). Characterisation of the zebrafish serotonin transporter functionally links TM10 to the ligand binding site. *J. Neurochem.* **105**, 1794-1805.
- Shi, L., Quick, M., Zhao, Y., Weinstein, H. and Javitch, J. A. (2008). The mechanism of a neurotransmitter:sodium symporter – inward release of Na⁺ and substrate is triggered by substrate in a second binding site. *Mol. Cell* **30**, 667-677.
- Silva, L. J. G., Pereira, A. M. P. T., Meisel, L. M., Lino, C. M. and Pena, A. (2015). Reviewing the serotonin reuptake inhibitors (SSRIs) footprint in the aquatic biota: uptake, bioaccumulation and ecotoxicology. *Environ. Pollut.* **197**, 127-143.
- Singh, S. K., Piscitelli, C. L., Yamashita, A. and Gouaux, E. (2008). A competitive inhibitor traps LeuT in an open-to-out conformation. *Science* **322**, 1655-1661.
- Singh, S. K., Yamashita, A. and Gouaux, E. (2007). Antidepressant binding site in a bacterial homologue of neurotransmitter transporters. *Nature* **448**, 952-956.
- Sloman, K. A., McDonald, M. D., Barimo, J. F., Lepage, O., Winberg, S., Wood, C. M. and Walsh, P. J. (2005). Does pulsatile urea excretion serve as a social signal in the gulf toadfish *Opsanus beta*? *Physiol. Biochem. Zool.* **78**, 724-735.
- Stoyek, M. R., Jonz, M. G., Smith, F. M. and Croll, R. P. (2017). Distribution and chronotropic effects of serotonin in the zebrafish heart. *Auton. Neurosci. Basic Clin.* **206**, 43-50.
- Sundin, L. I. (1995). Responses of the branchial circulation to hypoxia in the Atlantic cod, *Gadus morhua*. *Am. J. Physiol.* **268**, R771-R778.
- Sundin, L. and Nilsson, G. E. (2000). Branchial and circulatory responses to serotonin and rapid ambient water acidification in rainbow trout. *J. Exp. Zool.* **287**, 113-119.
- Sundin, L., Nilsson, G. E., Block, M. and Löfman, C. O. (1995). Control of gill filament blood flow by serotonin in the rainbow trout, *Oncorhynchus mykiss*. *Am. J. Physiol.* **268**, R1224-R1229.
- Taylor, J. R., Mager, E. M. and Grosell, M. (2010). Basolateral NBCe1 plays a rate-limiting role in transepithelial intestinal HCO₃⁻ secretion, contributing to marine fish osmoregulation. *J. Exp. Biol.* **213**, 459-468.
- Ullsch, G. R., Jackson, D. C. and Moallil, R. (1981). Metabolic oxygen conformity among lower vertebrates: the toadfish revisited. *J. Comp. Physiol. B* **142**, 439-443.
- Velarde, E., Delgado, M. J. and Alonso-Gómez, A. L. (2010). Serotonin-induced contraction in isolated intestine from a teleost fish (*Carassius auratus*): characterization and interactions with melatonin. *Neurogastroenterol. Motil.* **22**, e364-e373.
- Venugopalan, C. S., Holmes, E. P. and Kleinow, K. M. (1995). Evidence for serotonin involvement in the NAIMC excitatory neurotransmission in the catfish intestine. *J. Auton. Pharmacol.* **15**, 37-48.
- Wang, Y., Takai, R., Yoshioka, H. and Shirabe, K. (2006). Characterization and expression of serotonin transporter genes in zebrafish. *Tohoku J. Exp. Med.* **208**, 267-274.
- Wang, Y. and Walsh, P. J. (2000). High ammonia tolerance in fishes of the family Batrachoididae (Toadfish and Midshipmen). *Aquat. Toxicol.* **50**, 205-219.
- Wood, C., Hopkins, T. and Walsh, P. (1997). Pulsatile urea excretion in the toadfish (*Opsanus beta*) is due to a pulsatile excretion mechanism, not a pulsatile production mechanism. *J. Exp. Biol.* **200**, 1039-1046.
- Xu, F., Owen, J. and Frazier, D. T. (1995). Hypoxic respiratory responses attenuated by ablation of the cerebellum or fastigial nuclei. *J. Appl. Physiol.* **79**, 1181-1189.
- Yamashita, A., Singh, S. K., Kawate, T., Jin, Y. and Gouaux, E. (2005). Crystal structure of a bacterial homologue of Na⁺/Cl⁻-dependent neurotransmitter transporters. *Nature* **437**, 215-223.
- Yokoyama, T., Misuzu, Y.-Y. and Yamamoto, Y. (2013). Immunohistochemical localization of tryptophan hydroxylase and serotonin transporter in the carotid body of the rat. *Histochem. Cell Biol.* **140**, 147-155.
- Zhou, Z., Zhen, J., Karpowich, N. K., Goetz, R. M., Law, C. J., Reith, M. E. A. and Wang, D.-N. (2007). LeuT-desipramine structure reveals how antidepressants block neurotransmitter reuptake. *Science* **317**, 1390-1393.
- Zhou, Z., Zhen, J., Karpowich, N. K., Law, C. J., Maarten, E. A., Wang, D., Goetz, R. M., Law, C. J., Reith, M. E. A. and Wang, D.-N. (2009). Antidepressant specificity of serotonin transporter suggested by three LeuT-SSRI structures. *Nat. Struct. Mol. Biol.* **16**, 652-657.

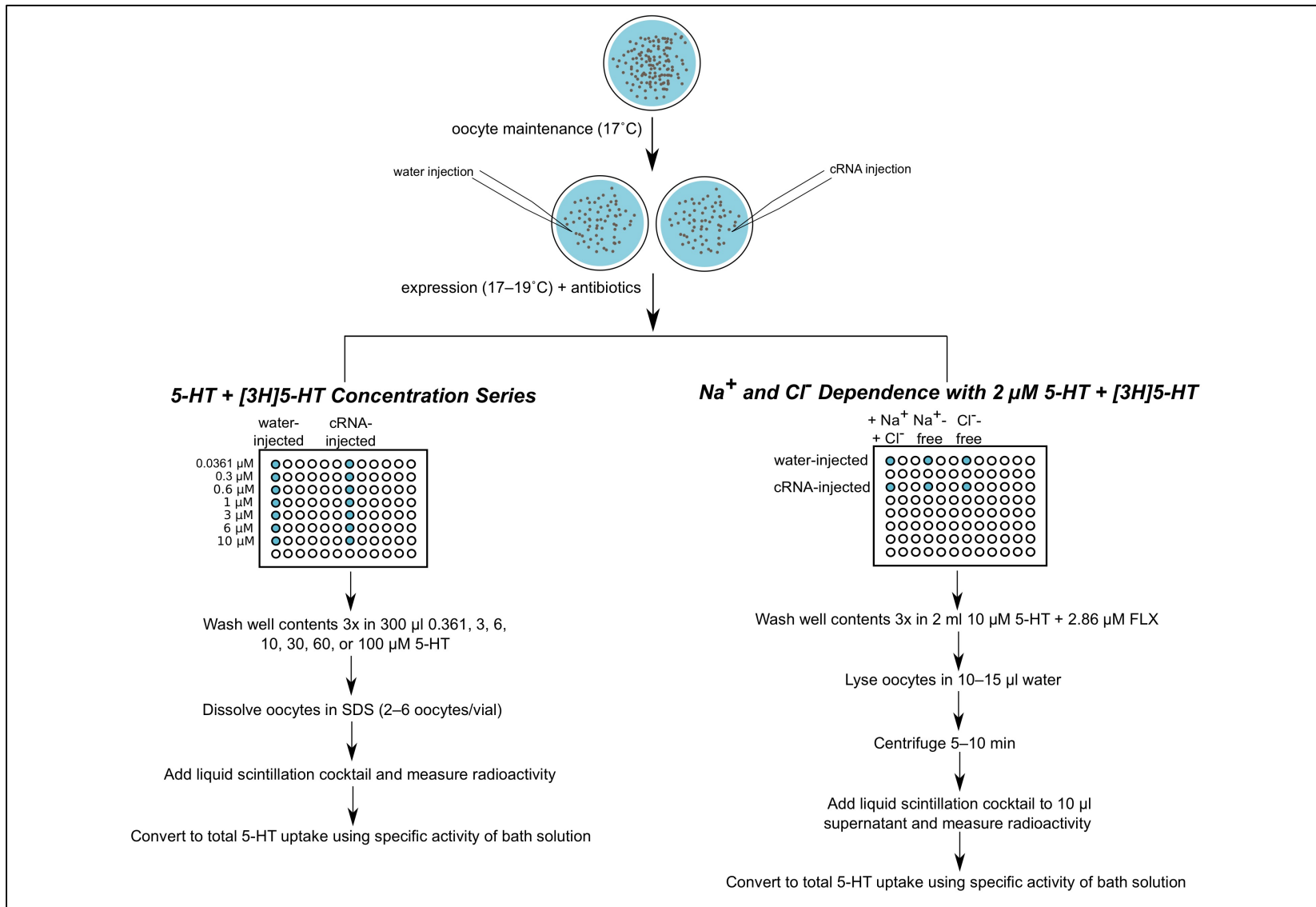


Figure S1: Transport kinetics experimental outline.