

Molecular and functional characterization of the Gulf toadfish serotonin transporter (SERT; SLC6A4)

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Summary Statement

In this study we present the sequence, structure, distribution, transport kinetics, and physiological significance of the serotonin transporter in a teleost fish.

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 reuptake inhibitors (SSRIs), a common group of antidepressants. The current study comprehensively assesses the sequence, tissue distribution, transport kinetics, and physiological function of a teleost SERT. The 2,022-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 $2.08 \pm 0.45 \mu\text{M}$, 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.

List of abbreviations: 5-hydroxytryptamine, 5-HT; serotonin transporter, SERT; selective serotonin reuptake inhibitor, SSRI; fluoxetine, FLX

1. 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; El-Merahbi et al., 2015; Pelster and Schwerte, 2012). 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 reuptake inhibitors (SSRIs), a common class of antidepressants including drugs like 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 about 600–630 amino acids (Blakely et al., 1991; Chang et al., 1996; Chen et al., 1998a; Chen et al., 1998b; Mortensen et al., 1999; Ramamoorthy et al., 1993). 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; Chang et al., 1996; Duncan et al., 1992; Kish et al., 2005; Lesch et al., 1993a). In the rest of the body, SERT has been documented in cardiovascular, respiratory, urinary, endocrine, skeletal, and digestive system tissues (Linder et al., 2009; Mortensen et al., 1999). 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; Severinsen et al., 2008; Wang et al., 2006). On a functional level, fish SERTs appear to possess a lower affinity for 5-HT than

mammalian SERTs (Severinsen et al., 2008; Wang et al., 2006), although they may be inhibited by SSRIs with similar effectiveness (Severinsen et al., 2008; Wang et al., 2006; reviewed by McDonald, 2017). SERT mRNA transcript has been documented both within the central nervous system and in peripheral tissues of teleost fish (Mennigen et al., 2010a; Norton et al., 2008; Wang et al., 2006), where it could be involved in such 5-HT-sensitive processes as catecholamine and cortisol secretion (Bernier and Perry, 1996; Fritsche et al., 1993; Lim et al., 2013; Medeiros and McDonald, 2012) or branchial (Forster et al., 1998; Fritsche et al., 1992; McDonald et al., 2010; Nilsson and Sundin, 1998; Sundin, 1995; Sundin et al., 1995), gastrointestinal (Buddington and Krogdahl, 2004; Kiliaan et al., 1989; Mori and Ando, 1991; Velarde et al., 2010; Venugopalan et al., 1995), or cardiovascular (Janvier et al., 1996; McDonald et al., 2010; Pellegrino et al., 2003) 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; Barkan et al., 2004; Marazziti et al., 1998) 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 (Caamaño-Tubío et al., 2007; Maurer-Spurej, 2005). It is therefore possible that another peripheral SERT location—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 since SSRIs are increasingly found as pollutants in aquatic environments, where they can potentially interfere with 5-HT signaling in exposed organisms (Barnes et al., 2008; Brooks et al., 2005; Kolpin et al., 2002; Ramirez et al., 2009); reviewed in (Brooks et al., 2003; McDonald, 2017; Silva et al., 2015). 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; compare it to SERT of other organisms; examine its tissue distribution; characterize its transport kinetics and ion dependence; and 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.

2. Materials and Methods

2.1 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 #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) to treat and prevent infection by ectoparasites. Fish were housed in aerated flow-through 20-gal aquaria supplied with filtered seawater from Biscayne Bay and were fed shrimp weekly to satiation. Water temperatures ranged from 20–24°C. All experimental protocols involving animals were approved by the University of Miami Institutional Animal Care and Use Committee.

2.2 Tissue collection, RNA isolation, DNase treatment, and cDNA synthesis

Fish were anaesthetized 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 collected as well, 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 RT-PCR (Thermo Fisher Scientific) using random hexamers.

2.3 Full-length sequence and phylogenetic analysis

Custom primers (Table 1) were designed based on the toadfish transcriptome (Schauer et al., 2017) and used in polymerase chain reaction (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).

2.4 Toadfish SERT expression in *Xenopus laevis* oocytes

2.4.1 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 resuspended 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 $\mu\text{g}\cdot\mu\text{l}^{-1}$ with nuclease-free water in preparation for injection into oocytes.

2.4.2 Oocyte injection and maintenance

Collagenase-treated, defolliculated oocytes (a kind gift from Dr. Gerhard Dahl, University of Miami, Miller School of Medicine, Miami, FL, USA) were maintained in antibiotic-free OR2 medium (in mM: 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 ($\sim 1 \text{ ng}\cdot\text{nl}^{-1}$) cRNA or equivalent volume MilliQ water (control group) using a Nanoliter 2000 Injector (World Precision Instruments, Sarasota, FL, USA). Post-injection, oocytes were maintained in ND96 medium (in mM: 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 $\cdot\text{ml}^{-1}$ penicillin, 10 mg $\cdot\text{ml}^{-1}$ streptomycin) at 17–19°C until assays were performed. Immediately before all assays, oocytes were rinsed twice in antibiotic-free ND96 medium.

2.4.3 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 nonradioactive 5-HT (as 5-HT hydrochloride; Sigma-Aldrich) and [³H]5-HT (as [³H]5-HT creatinine sulfate, 27.7 Ci $\cdot\text{mmol}^{-1}$; American Radiolabeled Chemicals, Saint 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 μM ; this concentration was chosen based on the K_m value of zebrafish SERT, estimated to be 2.13 μM and 4.2 μM 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 minutes was used for all experiments. To measure toadfish SERT transport kinetics, bath solutions were prepared with 7 different concentrations of [^3H]-5HT diluted with nonradioactive 5-HT to create a concentration series of 0.0361, 0.3, 0.6, 1, 3, 6, and 10 μM 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 μM 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 nonradioactive 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, 2–6 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 μM 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 2–6 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 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) 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 (v. 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, cpm) 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.

2.5 SERT mRNA distribution analysis with quantitative PCR (qPCR)

To analyze SERT transcript distribution among a suite of tissues, 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, Waltham, MA, USA) on an Mx3005P Multiple Quantitative PCR System (Stratagene, La Jolla, CA) with MxPro Software (v. 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, and 72°C for 30 s; and 95°C for 1 min. A dissociation curve was created from 60°C 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 Ct value, that Ct value was used. Data were analyzed using the $2^{-\Delta\Delta C_t}$ 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 (Mager et al., 2012; Medeiros et al., 2010). 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).

2.6 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 mM NaCl with 50 IU·ml⁻¹ sodium heparin; Sigma-Aldrich) as described previously (McDonald et al., 2000; Wood et al., 1997). 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 presample was first taken to flush the catheter, a 100-μl blood sample was collected, the pre-sample was reinjected 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 μg·g⁻¹ FLX (as fluoxetine hydrochloride; Toronto Research Chemicals) in warmed coconut oil (5 μl·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 analyzed for 5-HT concentrations using ELISA (ALPCO, Salem, NH, USA).

2.7 Statistics

Statistics were performed in Prism (v. 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} values. Uptake dependence on Na⁺

and Cl⁻ and SERT mRNA distribution in whole tissues was analyzed 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 analyze plasma 5-HT time course data. Statistically significant differences were defined as $p < 0.05$. Means are presented ± 1 standard error of the mean (s.e.m.).

3. Results

The full-length toadfish SERT protein-coding sequence (GenBank accession number ALK82492.1) is 2,022 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 *Larimicthys crocea*, diverging at only 65 amino acid residues (Fig. 2). Toadfish SERT is slightly less similar to zebrafish SERTA (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 μ M), 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 $2.08 \pm 0.45 \mu$ M (95% confidence interval [CI]: 0.84–3.33 μ M) and a V_{max} of 0.0095 ± 0.0009 pmol 5-HT·min⁻¹ (95% CI: 0.0070–0.01211 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 as compared to 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 5 of 8 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 about 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 ($0 \mu\text{g}\cdot\text{g}^{-1}$ FLX) and $1 \mu\text{g}\cdot\text{g}^{-1}$ FLX via IP injection did not result in significant changes in plasma 5-HT concentrations at any measured time point compared to pre-injection levels (Fig. 6A,B), while treatment with $10 \mu\text{g}\cdot\text{g}^{-1}$ FLX significantly increased plasma 5-HT by 1.6- to 1.7-fold from 30 min to 2 h after injection (Fig. 6C). In fish treated with the highest dose, $50 \mu\text{g}\cdot\text{g}^{-1}$ 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 ($p < 0.0001$).

4. 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 (Chang et al., 1996; Chen et al., 1998b; Ramamoorthy et al., 1993; 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 (Beuming et al., 2006; Celik et al., 2008; Marchler-Bauer et al., 2017; Quick et al., 2009; Shi et al., 2008; Singh et al., 2007; Singh et al., 2008; Yamashita et al., 2012; Zhou et al., 2007). 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 Na⁺:Cl⁻:K⁺:5-HT⁺) indicates that this process is electroneutral (Nelson and Rudnick, 1979; Rudnick and Nelson, 1978; 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 (Adams and DeFelice, 2003; Mager et al., 1994; Galli et al., 1997; Schicker et al., 2012; reviewed in De Felice, 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, fluoxetine also inhibits SERT-associated currents (Mager et al., 1994), raising the possibility that fluoxetine exposure could alter important ion-dependent processes like 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 ± 0.45 μM) is similar to reported K_m estimates for zebrafish SERTA (2.13 μM and 4.20 μM; Severinsen et al., 2008; Wang et al., 2006), while mammalian K_m estimates range from 0.28 to 0.70 μM (Chang et al., 1996; Chen et al., 1998b; Mortensen et al., 1999; Owens et al., 1997; Ramamoorthy et al., 1993). 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 (μM) and actual circulating concentrations (nM) are likely 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 fit our transport data better than the Michaelis-Menten model that has been used for other mammal and fish SERTs (Chang et al., 1996; Fontana et al., 2009; Mortensen et al., 1999; Severinsen et al., 2008; Wang et al., 2006). 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.

The discovery of SERT transcript throughout the toadfish brain is consistent with the widespread expression noted in previous studies on zebrafish and goldfish (Mennigen et al., 2010b; Norton et al., 2008). However, the markedly elevated SERT mRNA expression in the toadfish cerebellum relative to other brain regions is surprising when compared to other fish and mammals. While the goldfish cerebellum contains SERT transcript, levels do not appear higher than other brain regions using semiquantitative Northern blot (Mennigen et al., 2010), 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 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 to 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 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 (McDonald et al., 2011; Sloman et al., 2005), and are remarkably tolerant to a variety of environmental stressors including hypoxia (Hall, 1929; Ultsch et al., 1981) and ammonia (Wang and Walsh, 2000); therefore, it is 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 (Chang et al., 1996; Chen et al., 1998a; Lesch et al., 1993b; Mortensen et al., 1999; Ni et al., 2004; Pavone et al., 2008; Pavone et al., 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 to 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) (though 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.

While 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 filamental artery sphincter—a major site of blood flow control in the gill (Nilsson and Sundin, 1998; Pelster and Schwerte, 2012; Sundin and Nilsson, 2000). 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 (Caamaño-Tubío et al., 2007; Fänge, 1992; Mercado and Kilic, 2010). Since systemic inhibition of SERT with FLX does attenuate cardiovascular aspects of the toadfish hypoxia response (Panlilio et al., 2016) as well as the overall metabolic response to hypoxia in this fish (Amador et al., *submitted*), it is possible that cardiac SERT could play a specific role in the hypoxia response in hypoxia-tolerant species like toadfish (Hall, 1929; McDonald et al., 2010; Ultsch et al., 1981) and goldfish (reviewed in Bickler and Buck, 2007).

In addition to the heart, toadfish SERT mRNA expression was also elevated in the liver compared to several other tissues. SERT transcript has likewise been found in the liver of goldfish, though 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. Additionally, the liver may play a specific role 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 (McDonald et al., 2011; Morando et al., 2009) suggests the presence of SERT-mediated 5-HT clearance from the blood. Since 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 ($1 \mu\text{g}\cdot\text{g}^{-1}$) of FLX (comparable to the upper limit of typical therapeutic doses in humans; see McDonald, 2017), a moderate increase was evident at $10 \mu\text{g}\cdot\text{g}^{-1}$ FLX, and a dramatic and rapid increase over several time points was measured at $50 \mu\text{g}\cdot\text{g}^{-1}$ 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 ($50 \mu\text{g}\cdot\text{g}^{-1}$) 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 (McDonald et al., 2011; Morando et al., 2009). However, the implants in previous 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, likely 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 minutes. 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 regards 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 herein 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.

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6. Competing Interests

The authors declare no competing interests.

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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

Figures

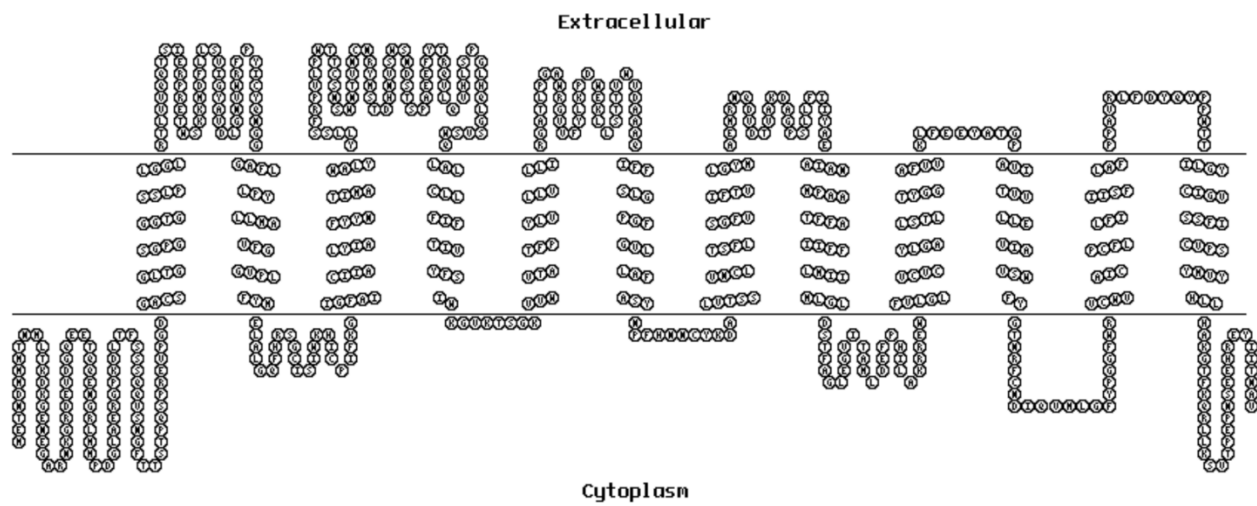


Figure 1: Predicted secondary structure and membrane organization of the toadfish SERT.

Figure generated using MEMSAT2 software.

Toadfish 1 METNDMMNMLTKDKGENEGARNKGRDEVDCQEE-TQ--QENGRIMMPDGLAERGPDKLTFSSSQVSN
Yellow_Croaker 1 METKDMMMMSMLMDKEENEGERDKAREELGGEQEGTQ--QENGRIMLADGLTEKGPKSLSGSGGQVSN
Zebrafish_A 1 ---MDMKESMMNOEY-----GGEQQVPESQENGRILVV-DSVPEKDKSGSGPG--QVSN
Rat 1 METTPNSQKVLSECK-----DREDC--QENG--VLQKGVPTTADRA--EPS--QISN
Human 1 METTPNSQKQLSACE-----DGEDC--QENG--VLQKVVPTEGDKV--ESG--QISN

Toadfish 68 GFTTSTPQSPREVPFGDGACSGLTGSGPGGCTGSSSLPLGLRLTLVQQTSIERPRETWSKKMDFLLSVIGY
Yellow_Croaker 69 GFTTSTPQSPREGBGTADSSRD TASGPGGCTGSSVPPGSLRLTLVQQTSIERPRETWSKKMDFLLSVIGY
Zebrafish_A 51 GYRSTSPQSPKEGAGT-----CTDRVNTPGTFRLTLVQQTSLDPPRETWSKKMDFLLSVIGY
Rat 46 GYSAV----PSTSGAD-----EASHSIPATTTLVAEIRQGE--RETWKKMDFLLSVIGY
Human 46 GYSAV----PSPGAGD-----DTRHSIPATTTLVAEHLQGE--RETWKKKDFLLSVIGY

Toadfish 138 AVDLGNVWRFPYICYQNGGGAFLLPYLLMAVFGVPLFYMEALALGQFHRSGCISIWKKHCPIFKGIGFAI
Yellow_Croaker 139 AVDLGNVWRFPYICYQNGGGAFLLPYLLMAVFGVPLFYMEALALGQFHRSGCISIWKKHCPIFKGIGFAI
Zebrafish_A 108 AVDLGNVWRFPYICYQNGGGAFLLPYLLMAVFGVPLFYMEALALGQFHRSGCISIWKKHCPIFKGIGFAI
Rat 96 AVDLGNIWRFPYICYQNGGGAFLLPYTIMATFGGTPLFYMEALALGQVHRNGCISIWKKHCPIFKGIGYAI
Human 96 AVDLGNVWRFPYICYQNGGGAFLLPYTIMATFGGTPLFYMEALALGQVHRNGCISIWKKHCPIFKGIGYAI

Toadfish 208 CIIALYIAFYNTIMAWALYYLISSEFPVLPWTTCSNSWNTVNCNRYMSTDHNVSWNSNSTSPAEEFYTR
Yellow_Croaker 209 CIIALYIAFYNTIMAWALYYLISSEQPTLPWSTCTNSWNTCTCNRYMSTDHNVSWNSNSTSPAEEFYTR
Zebrafish_A 178 CIIALYIAFYNTIMAWALYYLISSEPRATLPWTTCTNNRWNTNCTHYLSTDLNVSWNTNSISPAEEFYVR
Rat 166 CIIAFYIASYNTIIMAWALYYLISSELDRLPWTCTNSWNTGNCTNYFAOD-NITWTLHSTSPAEEFYLR
Human 166 CIIAFYIASYNTIMAWALYYLISSETDQLPWTSCKNWNTGNCTNYFESD-NITWTLHSTSPAEEFYTR

Toadfish 278 QVLQVHLSPLGHQLGSGVSWQLALCLLFIFTIVYFSIWKGVKTSKGKVVWVTATFPYLVLLVLLVRGATLPG
Yellow_Croaker 279 QVLQVHLSPLGHQLGSGVSWQLALCLLFIFTIVYFSIWKGVKTSKGKVVWVTATFPYLVLLVLLVRGATLPG
Zebrafish_A 248 QVLQVHLSPLGHQLGSGVSWQLALCLLFIFTIVYFSIWKGVKTSKGKVVWVTATFPYLVLLVLLVRGATLPG
Rat 235 HVLOTHQSGLODLGTTISWQLALCLIVLIFTIVYFSIWKGVKTSKGKVVWVTATFPYLVLLVLLVRGATLPG
Human 235 HVLOTHRSKGLQDLGGTSWQLALCLIMLIFTIVYFSIWKGVKTSKGKVVWVTATFPYLVLLVLLVRGATLPG

Toadfish 348 AWRGVVFYLPKPDWQKLLSTTVWVDAAQIFFSLGPGFGVLLAFASYNPFHNNCYKDALVTSSVNCITSFL
Yellow_Croaker 349 AWRGVVFYLPKPDWQKLLSTTVWVDAAQIFFSLGPGFGVLLAFASYNPFHNNCYKDALVTSSVNCITSFL
Zebrafish_A 318 AWRGVVFYLPKPDWQKLLSTTVWVDAAQIFFSLGPGFGVLLAFASYNPFHNNCYKDALVTSSVNCITSFL
Rat 305 AWRGVVFYLPKPNWQKLLSTTVWVDAAQIFFSLGPGFGVLLAFASYNPFHNNCYKDALVTSSVNCITSFV
Human 305 AWRGVVFYLPKPNWQKLLSTTVWVDAAQIFFSLGPGFGVLLAFASYNPFHNNCYKDALVTSSVNCITSFV

Toadfish 418 SGFVIFTVLGYMAEMRNQDVEVAKDAGPSLLFIIYAEAIANMPAATFFAIIFFLMIIMLGLDSTFAGLE
Yellow_Croaker 419 SGFVIFTVLGYMAEMRNQDVEVAKDAGPSLLFIIYAEAIANMPAATFFAIIFFLMIIMLGLDSTFAGLE
Zebrafish_A 388 SGFVIFTVLGYMAEMRNQDVEVAKDAGPSLLFIIYAEAIANMPAATFFAIIFFLMIIMLGLDSTFAGLE
Rat 375 SGFVIFTVLGYMAEMRNQDVEVAKDAGPSLLFIIYAEAIANMPAATFFAIIFFLMIIMLGLDSTFAGLE
Human 375 SGFVIFTVLGYMAEMRNQDVEVAKDAGPSLLFIIYAEAIANMPAATFFAIIFFLMIIMLGLDSTFAGLE

Toadfish 488 GVITAMLDEFPHILAKRREWFVLGLVCVCYLGAISTLTYGGAFFVVKLFEEYATGPAVITVVLLEVIASVW
Yellow_Croaker 489 GVITAMLDEFPHILAKRREWFVLGLVCVCYLGAISTLTYGGAFFVVKLFEEYATGPAVITVVLLEVIASVW
Zebrafish_A 458 GVITAMLDEFPHILAKRREWFVLGLVCVCYLGAISTLTYGGAFFVVKLFEEYATGPAVITVVLLEVIASVW
Rat 445 GVITAMLDEFPHILAKRREWFVLGLVCVCYLGAISTLTYGGAFFVVKLFEEYATGPAVITVVLLEVIASVW
Human 445 GVITAMLDEFPHILAKRREWFVLGLVCVCYLGAISTLTYGGAFFVVKLFEEYATGPAVITVVLLEVIASVW

Toadfish 558 FYGTNRFCNDIQVMLGFYPGFWRVWCVAICPCFLLFIIISFLAFPPEVRLFDYQYPPWTTILGYCIGVS
Yellow_Croaker 559 FYGTNRFCNDIQVMLGFYPGFWRVWCVAICPCFLLFIIISFLAFPPEVRLFNHYPPWTTILGYCIGVS
Zebrafish_A 528 FYGTNRFCNDIQVMLGFYPGFWRVWCVAICPCFLLFIIISFLAFPPEVRLFDYQYPPWTTILGYCIGVS
Rat 515 FYGTNRFCNDIQVMLGFYPGFWRVWCVAICPCFLLFIIISFLAFPPEVRLFDYQYPPWTTILGYCIGVS
Human 515 FYGTNRFCNDIQVMLGFYPGFWRVWCVAICPCFLLFIIISFLAFPPEVRLFDYQYPPWTTILGYCIGVS

Toadfish 628 SFICVPSYMYVHLHAKGTFKQRLLSITPE-P-NSEHRE-YIITNAV
Yellow_Croaker 629 SFICVPSYMYVHLHAKGTFKQRLLSITPE-P-SSDQHRD-LIVTNAV
Zebrafish_A 598 SFICVPSYMYVHLHAKGTFKQRLLSITPE-P-SSDQHRD-LIVTNAV
Rat 585 SVICPTPTIYRLISTPGTLKBRITKSITPETP-TEIPCGD--IRLNAV
Human 585 SFICPTPTIYRLISTPGTLKBRITKSITPETP-TEIPCGD--IRLNAV

Figure 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 carats (^) denote residues likely important for SSRI binding (Zhou et al., 2009).

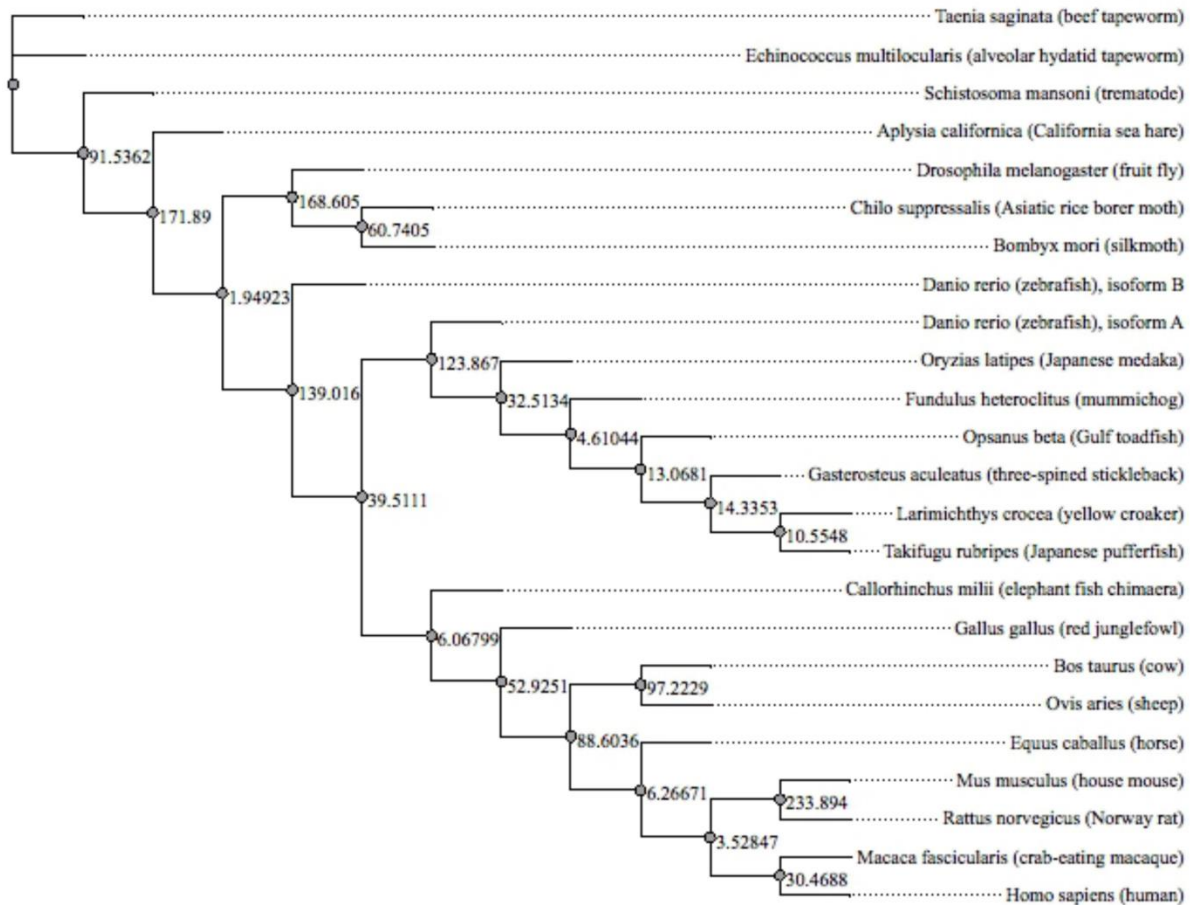


Figure 3: Phylogenetic analysis of amino acid sequences. Genbank/RefSeq/Ensembl accession numbers, from top: OCK35329.1, CDS36751.1, ABK51389.1, NP_001191502.1, NP_523846.2, AKL78871.1, NP_001037436.1, AAI63371.1, AAI63777.1, XP_004075963.2, JAR86319.1, ALK82492.1, ENSGACP000000008195, KKF08838.1, XP_003968295.2, AFO94871.1, AAS79016.1, AAD26262.1, NP_001274253.1, 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.

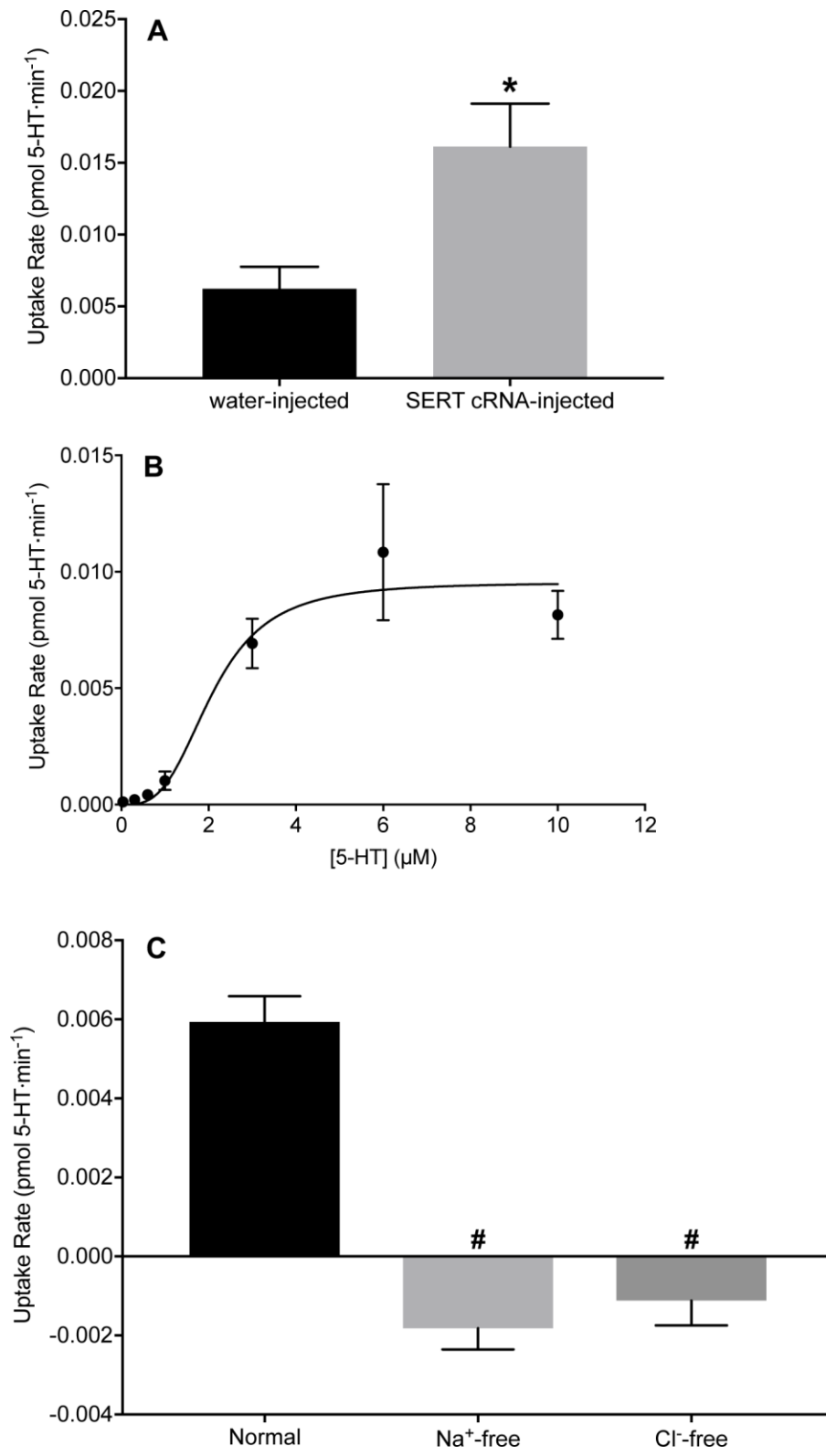


Figure 4: SERT-mediated 5-HT uptake in *Xenopus laevis* oocytes. (A) 5-HT uptake in cRNA-injected oocytes *versus* those injected with water (controls). An asterisk (*) denotes a statistically significant difference ($p < 0.05$, Student's t-test, $n = 7$ [cRNA-injected] or 8 [controls]). (B) SERT 5-HT 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. An octothorpe (#) denotes 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).

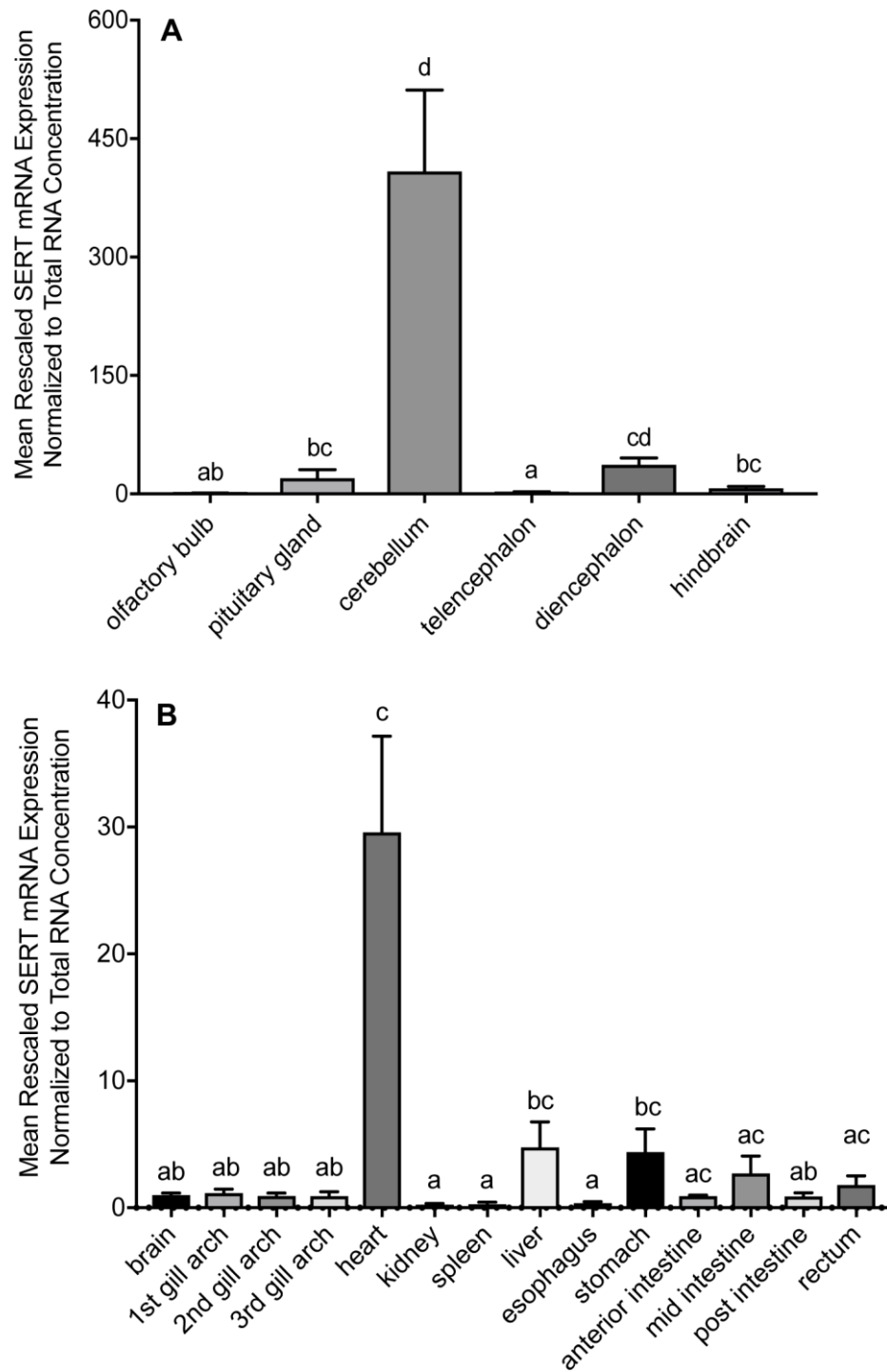


Figure 5: Mean SERT mRNA expression in (A) toadfish brain segments and (B) a suite of toadfish tissues. Expression is normalized to the total RNA concentration used in cDNA synthesis and rescaled relative to expression in the (A) olfactory bulb or (B) brain. Bars not

sharing a 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).

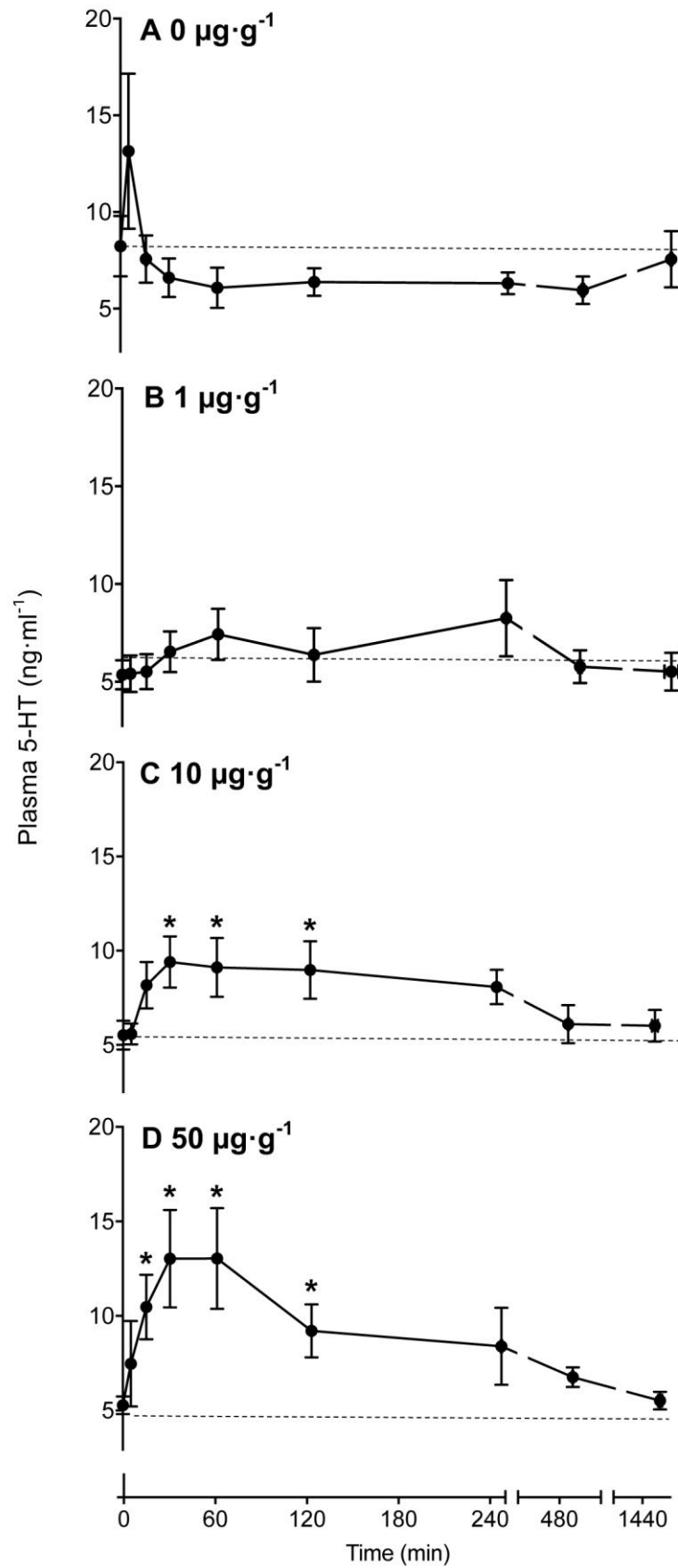


Figure 6: Plasma 5-HT concentrations over time after SERT inhibition by FLX. (A) $0 \mu\text{g} \cdot \text{g}^{-1}$ FLX, (B) $1 \mu\text{g} \cdot \text{g}^{-1}$ FLX, (C) $10 \mu\text{g} \cdot \text{g}^{-1}$ FLX, (D) $50 \mu\text{g} \cdot \text{g}^{-1}$ FLX. An asterisk (*) denotes a statistically significant difference from time $t = 0$ ($p < 0.05$; two-way repeated measures ANOVA with Dunnett's multiple comparisons test; $n = 7$).

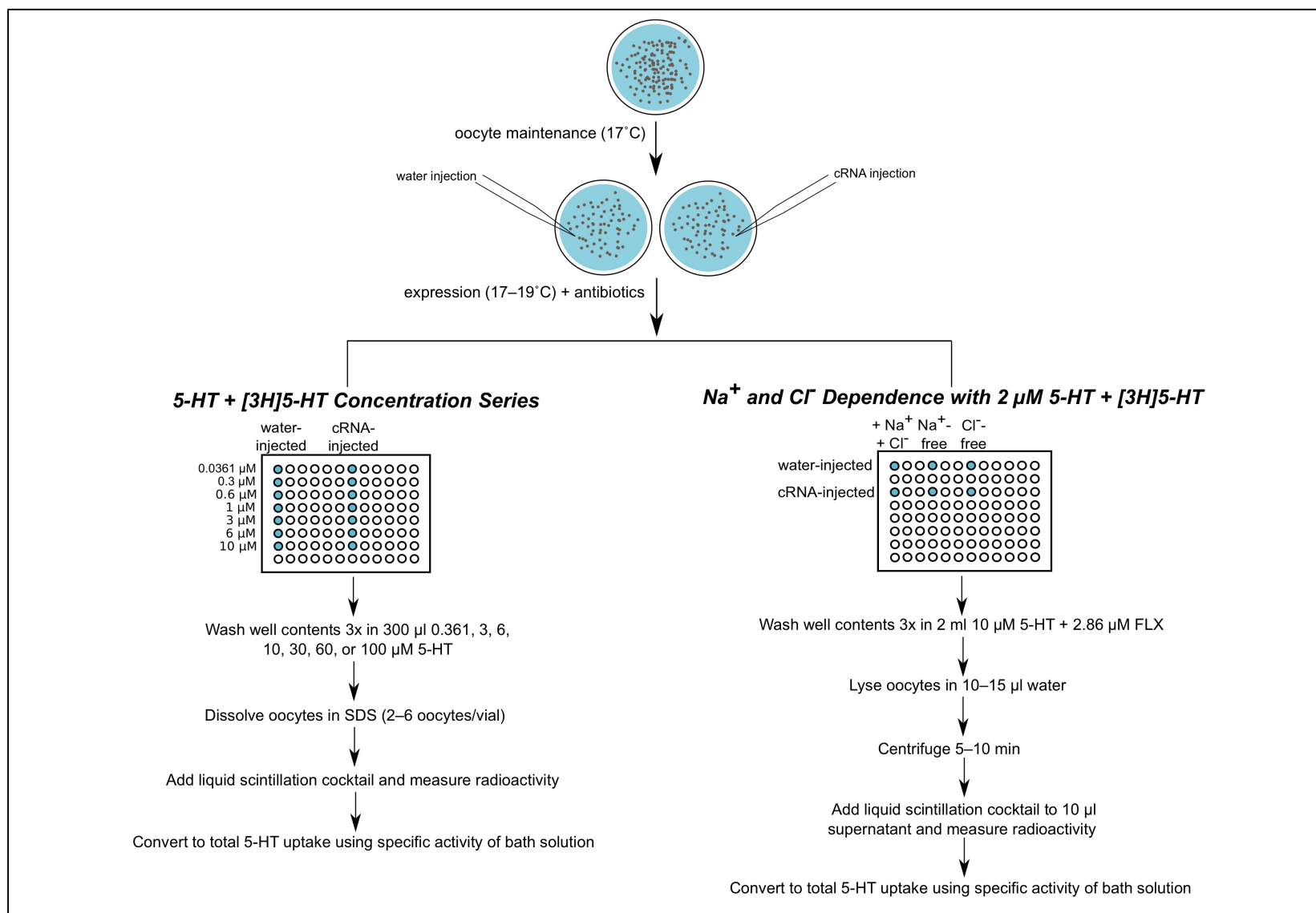


Figure S1: Transport kinetics experimental outline.