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***In Vivo* Investigation of Escitalopram's Allosteric Site on the Serotonin Transporter**

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Abstract

Escitalopram is a commonly prescribed antidepressant of the selective serotonin reuptake inhibitor class. Clinical evidence and mapping of the serotonin transporter (SERT) identified that escitalopram, in addition to its binding to a primary uptake-blocking site, is capable of binding to the SERT via an allosteric site that is hypothesized to alter escitalopram's kinetics at the SERT. The studies reported here examined the *in vivo* role of the SERT allosteric site in escitalopram action. A knockin mouse model that possesses an allosteric-null SERT was developed. Autoradiographic studies indicated that the knockin protein was expressed at a lower density than endogenous mouse SERT (approximately 10–30% of endogenous mouse SERT), but the knockin mice are a viable tool to study the allosteric site. Microdialysis studies in the ventral hippocampus found no measurable decrease in extracellular serotonin response after local escitalopram challenge in mice without the allosteric site compared to mice with the site ($p = 0.297$). In marble burying assays there was a modest effect of the absence of the allosteric site, with a larger systemic dose of escitalopram (10-fold) necessary for the same effect as in mice with intact SERT ($p = 0.023$). However, there was no effect of the allosteric site in the tail suspension test. Together these data suggest that there may be a regional specificity in the role of the allosteric site. The lack of a robust effect overall suggests that the role of the allosteric site for escitalopram on the SERT may not produce meaningful *in vivo* effects.

Keywords

Serotonin transporter; allosteric; SSRI; marble burying; tail suspension test; escitalopram

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1. Introduction

Selective serotonin reuptake inhibitors (SSRIs²) are a drug class frequently prescribed to patients with major depressive disorder, generalized anxiety disorder, obsessive-compulsive disorder, panic disorder or other psychiatric disorders (Chapman *et al.*, 2005; Katon *et al.*, 2007; Strine *et al.*, 2008). SSRIs act by disrupting the serotonin transporter's (SERT) movement of serotonin into cells, which results in greater serotonin accumulation outside of cells. Despite a similar general mechanism of action and overall efficacy, there is a variety of patient response within and between individual SSRIs (Nierenberg *et al.*, 2010; Blier, 2013). A clearer understanding of the differences between SSRIs may benefit future effective drug development and patient treatment. These differences may include identification of new drug targets to treat patients or to augment current treatments.

Of current interest is an allosteric binding site for several SSRIs on the SERT, initially identified in studies of escitalopram's mechanism of action. Escitalopram is the *S*-enantiomer of the racemic SSRI citalopram. Its partner enantiomer, *R*-citalopram, is considered to have no clinical value as an SSRI, because its affinity to inhibit the SERT is 30-fold less than escitalopram (Owens *et al.*, 2001). Consequently, escitalopram was developed as an enantiopure compound. In early clinical trials comparing escitalopram to citalopram, patients were given equimolar doses of the *S*-enantiomer and escitalopram treatment was, surprisingly, found to be superior to the matched citalopram dose (Gorman *et al.*, 2002; Auquier *et al.*, 2003; Lepola *et al.*, 2003, 2004).

A distinct second binding site for escitalopram on the human SERT (hSERT) was subsequently identified and this putative allosteric site was hypothesized to be the mechanism by which escitalopram was superior to citalopram in the clinic (Larsen *et al.*, 2004; Elfving and Wiborg, 2005; Neubauer *et al.*, 2006). *In vitro* work noted that escitalopram stabilized its own complexes with the hSERT, an effect that was diminished in the presence of *R*-citalopram (Chen, Larsen, Neubauer, *et al.*, 2005; Plenge *et al.*, 2007). The observed changes in dissociation and association kinetics are not predicted by simple changes in escitalopram's fractional occupancy at the primary binding site upon the addition of *R*-citalopram, and these kinetic data support the role of an allosteric site. Further, escitalopram is also able to alter dissociation of other SSRIs, and *R*-citalopram also slows dissociation of SSRIs, including paroxetine, sertraline and fluoxetine, from the hSERT, but not to the extent of that produced by escitalopram (Chen, Larsen, Sánchez, *et al.*, 2005).

Preclinical work in rodents also replicated the blunting effect of *R*-citalopram on escitalopram effects in serotonin efflux, ultrasonic vocalization, and elevated plus maze studies to name a few (Mørk *et al.*, 2003; Sánchez *et al.*, 2003; Sánchez, 2006). Specifically in rats, microdialysis experiments determined that while escitalopram evokes a reliable response of increased extracellular serotonin, the addition of *R*-citalopram blunts the escitalopram response in a dose-dependent manner (Mørk *et al.*, 2003). As an antagonist of the SERT, though with a low affinity, it would be expected that the addition of *R*-citalopram

²Abbreviations: AUC, area under the curve; ED₅₀, median effective dose; HPLC, high-performance liquid chromatography; hSERT, human serotonin transporter; MB, marble burying; mSERT, mouse serotonin transporter; SERT, serotonin transporter; SSRI, selective serotonin reuptake inhibitor; TST, tail suspension test

would be minimal or slightly additive rather than significantly reductive. This finding was in line with the early clinical observations of escitalopram's superiority.

The observed allosteric activity *in vitro* was hypothesized to underlie the preclinical observations. In the present study these two phases are combined to directly examine the *in vivo* role of the allosteric site using a knockin mouse model that has the hSERT with or without the allosteric site. Very recent work using another allosteric-null hSERT knockin mouse investigated the interaction of escitalopram and *R*-citalopram at the allosteric site. That work concluded that the allosteric site is not a locus of *R*-citalopram antagonism of escitalopram (Jacobsen *et al.*, 2014). Here the role of escitalopram alone at the allosteric site is investigated to identify if the presence or absence of the allosteric site alone is enough to alter escitalopram's potency. We examined knockin mice fully humanized at the SERT, but with different sequences at the proposed allosteric site, using *in vivo* microdialysis with localized escitalopram exposure and two behavioral assays. The goal of these studies was to identify if the absence of the allosteric site altered the amount of escitalopram necessary to see the same level of response. We characterized the knockin protein expression via autoradiography, as well, to determine if protein expression (pattern and density) was altered. We hypothesized that in mice with allosteric-null hSERT, a larger escitalopram dose would be necessary to achieve the response seen at a lower dose for mice with intact hSERT.

2. Materials and Methods

2.1 Knockin Mice

Mice with either the hSERT protein (hSERT-wt) or mutant allosteric-null hSERT protein with 6 amino acid substitutions: I522V, I553T, M558S, S559N, S574T, I575T (hSERT-mut) were generated via homologous recombination and established on a 129S6/SvEv background as previously described for the hSERT-wt mice and similar, albeit distinct, allosteric-null mutant (Jacobsen *et al.*, 2014). The substitutions on the hSERT-mut protein significantly reduce escitalopram's *in vitro* allosteric activity, and it is important to note that this mutation is more effective at eliminating the allosteric site than that of the Jacobsen group as that these mutated amino acids are key to allosteric binding (Neubauer *et al.*, 2006). These mice were heterozygous for either the hSERT-wt or hSERT-mut gene targeted replacement of the mouse SERT gene. The mice were backcrossed to C57BL/6J over 10 generations and then bred until fully humanized at the SERT locus with no mouse SERT (mSERT) present.³ The mice were maintained on the C57BL/6J background and bred using heterozygous hSERT-wt/mut mice. The presence or absence of the hSERT-wt or hSERT-mut alleles was identified via genotype specific primers using standard PCR methods. Mice were group housed by sex, 3–5 mice per cage with *ad libitum* access to food and water with a 12 hour light:dark cycle. Male mice were used in all experiments and female mice were used as well in the autoradiography and behavioral experiments with no noted sex effects. Wildtype male C57BL/6J mice (Jackson Labs, Bar Harbor, ME) were used for the

³The hSERT knockin mice have been deposited to Jackson Labs and are available as follows:
hSERT-wt: C57BL/6-Slc6a4^{tm1}(SLC6A4)^{Kres}/J stock 011088
hSERT-mut: C57BL/6-Slc6a4^{tm2}(SLC6A4)^{Kres}/J stock 011089 animals'

autoradiography and referred to as mSERT mice. All experiments were conducted in the light cycle in accordance with an approved protocol from Emory University's Animal Care and Use Committee and follow the Guide for the Care and Use of Laboratory Animals

2.2 Drugs

Escitalopram oxalate was a generous gift from H. Lundbeck USA (Paramus, NJ). [^3H]-citalopram was obtained from Amersham (Piscataway, NJ). Fluoxetine HCl and fluvoxamine maleate were obtained from Sigma-Aldrich (St. Louis, MO). All drugs administered to animals were dissolved in sterile saline.

2.3 Autoradiography

To make comparisons between the hSERT-wt, hSERT-mut, and endogenous wildtype mouse SERT (mSERT) expression in mouse brains, tissue was collected from mice of each of those homozygous genotypes, as well as from heterozygous hSERT-wt/mut mice. Both male and female mouse brains were included so that both sexes could be characterized, although only male mice were included for the mSERT group. Mice were euthanized with an overdose of isoflurane and then quickly decapitated. Immediately the brain was extracted and fresh frozen on dry ice. All brains were stored at $-80\text{ }^{\circ}\text{C}$ until use. Brains were sliced at $-16\text{ }^{\circ}\text{C}$ on a cryostat at $20\text{ }\mu\text{m}$ thick slices. Serial sections were distributed across four series, with 4 slices obtained at the level of the frontal cortex, hippocampus and raphe nucleus. Multiple series of the slides allowed for total binding and non-specific binding to be performed in the same brains at near identical slices. Regions were identified according to Franklin and Paxinos's *The Mouse Brain in Stereotaxic Coordinates* (Franklin and Paxinos, 1997). Frontal cortex slides were at the atlas's Figure 22, hippocampus slides at Figure 47, and raphe nucleus slides at Figure 68. Slices were fixed on Fisherbrand Superfrost/Plus Microscope Slides (Pittsburgh, PA), and were stored at $-80\text{ }^{\circ}\text{C}$ until use.

The radioligand for the autoradiography was [^3H]-citalopram. Brain slices were thawed to room temperature then preincubated in SERT buffer (52.2 mM Tris HCl, 126.4 mM NaCl, 5.26 mM KCl) for 15 minutes. Total binding and non-specific binding solutions were also made in SERT buffer. The total binding solution was 2 nM [^3H]-citalopram, and the non-specific binding solution was 2 nM [^3H]-citalopram with 1 μM non-radiolabeled escitalopram. The slides incubated in either the total binding or non-specific binding solutions for 60 minutes. The slides were then washed 2 times for 10 minutes each in $0\text{ }^{\circ}\text{C}$ SERT buffer to end the exposure to the radioligand and in the case of the non-specific binding the competitive escitalopram. Slides were then dipped in cool dH_2O and finally dried under a cool stream of air. Once the slides were fully dry, they were placed in a cassette and exposed to Kodak Biomax MR Film (Sigma-Aldrich, St. Louis, MO) for 13 weeks before development. A tritium standard was included in the cassette for quantitative measurements.

The NIH's ImageJ was used to make quantitative densitometric measurements (Rasband, 2014). For each mouse a single slice at each the frontal cortex, hippocampus, and raphe nucleus was analyzed. For each region a free-hand shape was drawn to outline the region, and ImageJ determined a density which was then converted to nCi/mg based on the tritium

standard curve. Non-specific binding was subtracted from total binding, and then analysis was performed using IBM's SPSS (Armonk, NY). A 1-way ANOVA was used to compare the nCi/mg values of SERT expression across the four genotypes of hSERT-wt, hSERT-mut, hSERT-wt/mut, and mSERT.

2.4 Microdialysis

Microdialysis was modified from a previously published method of ours which allows for microdialysis sampling as well as retrodialysis application of drug in the same location (Thrivikraman *et al.*, 2013). Pilot studies were completed to determine escitalopram concentrations to use as well as to confirm the use of fluoxetine as an allosteric-inactive SSRI control. The final concentration of escitalopram and fluoxetine was the same: 0.0462 μ M as a low concentration and 0.1 μ M as a high concentration, a one-third log₁₀ unit separation. These concentrations empirically showed that both concentrations evoked a serotonin response above baseline levels. There was no consistent response to drug below this concentration range.

All microdialysis studies were completed in anesthetized mice, and the entire procedure lasted 8 hours, with the mouse being decapitated before recovering from anesthesia so that its brain could be collected to confirm probe placement. Mice were anesthetized with urethane (Sigma-Aldrich, St. Louis, MO), at 1.6 g/kg delivered in a volume of 15 μ L/g. Each mouse was weighed so that a proper dose of urethane was delivered. Once the mouse failed to react to a firm toe-pinch, it was secured in the stereotactic frame and the skull exposed through the skin. Relative to bregma, a hole was made in the skull at AP = -3.2 and ML = -2.5. The tip of the microdialysis probe was then inserted to DV = -4.0. The coordinates of AP = -3.2, ML = -2.5, and DV = -4.0 placed the probe within the ventral hippocampus, approximately at Figure 57 in *The Mouse Brain in Stereotaxic Coordinates* (Franklin and Paxinos, 1997). The probe was then secured in place using dental cement. After the cement was firm and dry the mouse was carefully placed on a heating pad for the remaining duration of the microdialysis procedure.

The microdialysis probe was a CMA 11 (Harvard Apparatus, Holliston, MA). The cuprophane probe had a 6 kDa cutoff. The probe length was 2 mm. Prior to insertion, the microdialysis probe was primed overnight in artificial cerebrospinal fluid (aCSF, 142 mM NaCl, 2.95 mM KCl, 1.36 mM CaCl₂, 0.98 mM MgCl₂, 0.99 mM Na₂HPO₄, 0.22 mM NaH₂PO₄). Once the probe began priming the flow rate continued at 1 μ L/min until the experiment was completed, and the probe was attached to the microdialysis system the entire time.

The microdialysis system was identical to that we have described previously (Thrivikraman *et al.*, 2013). In brief, a 6-port valve (Upchurch, Oak Harbor, WA) was used to regulate the switch between a flow of aCSF or aCSF with SSRI for retrodialysis. The valve allows for two simultaneous pathways. With a flow rate of 1 μ L/min, every 20 minutes a sample was collected of a volume of 20 μ L. This allowed a collected sample to match the exact exposure time of the solution filled into the loop. This allows for a single sample to match the exposure to either escitalopram or fluoxetine. Samples were collected into HPLC (high-

performance liquid chromatography) vials and were immediately capped and stored at -80°C until analysis.

A schematic of the experimental timeline is found in Figure 1A. After the microdialysis probe is inserted into the mouse's ventral hippocampus, there is an equilibration and recovery period of 80 minutes (4 samples). After the recovery, time was set to 0 minutes and the first baseline sample was labeled 0 minutes (consequently sample collection lasted from -20 to 0 minutes). Four baseline samples were collected, at 20 minutes each, a pattern maintained for the duration of the experiment. The first drug challenge sample was from 60–80 minutes, and labeled sample 80 minutes. The second drug challenge was from 180–200 minutes, and labeled sample 200 minutes. A third challenge from 300–320 minutes was of a high potassium concentration (called high K^{+} , aCSF with 10X KCl [29.5 mM]). This high K^{+} challenge was to determine the integrity of the tissue. A poor high K^{+} response was criteria for removing the mouse from the data set. Each mouse received a low concentration followed by a high concentration of the either escitalopram or fluoxetine, and no mouse received both SSRIs.

After the probe was removed, the mouse was decapitated, its brain removed, and fresh frozen on dry ice. The brain was stored at -80°C for later analysis of proper probe placement. Any misplaced probe resulted in removal of that mouse from the data set. Any mouse that died during the microdialysis experiment was also excluded.

HPLC was used to measure the serotonin in the microdialysis samples. All samples per individual mouse were analyzed on a single batch run on the HPLC with appropriate standards. The serotonin standards were 0.1, 0.2, 1, 2, and 5 nM, and were used to create a standard curve to quantify the serotonin in each sample.

The HPLC system was composed of C18 column (ESA, ThermoScientific, Sunnyvale, CA), dimensions of 150 X 3.2 mm with a 3 μm particle size and 120 Å pore size. MD-TM mobile phase (90 mM NaH_2PO_4 , 50 mM monohydrate citric acid, 1.7 mM 1-octanesulfonic sodium salt, 50 μM EDTA, 10% acetonitrile, pH = 3, made in ddH₂O) was delivered through the system via an ESA 528 solvent delivery pump, set at a flow rate of 0.600 mL/min. Samples were loaded into the ESA Model 542 refrigerated autosampler, 7 $^{\circ}\text{C}$. The autosampler loaded 15 μL of the individual 20 μL dialysate sample onto the column. An ESA Coulochem III detector with a model 5040 ESA dual-channel analytical cell and model 5020 ESA guard cell were used for electrochemical analysis. EZChrom Elite software (Scientific Software, Agilent Technologies, Santa Clara, CA) on a desktop computer collected the data and created the chromatograms. The EZChrom Elite software was also used to analyze and quantify the data using the created standard curve for each batch run.

Data for each mouse were analyzed using the area under the curve (AUC) for a plot of serotonin concentration over time. Prism 6.0 (GraphPad, La Jolla, CA) was used to plot raw serotonin concentrations over time. Prism then determined the AUC for the first two time points of drug exposure: samples ending at 80 and 100 minutes for the low concentration, and samples ending at 200 and 220 minutes for the high concentration. The AUC data were then analyzed as the percent change from baseline data: SPSS (IBM) was used to run a 2-

way ANOVA to determine the genotype X drug effect for each the low and high drug concentration.

There were 8 mice in each of the 4 experimental groups (2 genotypes X 2 SSRIs). One hSERT-wt mouse in the fluoxetine group was excluded due to improper probe placement, resulting in only 7 mice in that group. Male mice 8–12 weeks old were used for the microdialysis experiments. Due to the intensive nature of these experiments, female mice were not included. Female mice would have been included to examine potential sex differences should these initial experiments have proven more conclusive.

2.5 Marble Burying

The MB assay can be performed a number of ways, all with the same general pattern of introducing a number of items that an animal can bury during a set period of time. Here, the MB session included 20 black marbles of 1 centimeter diameter arranged in a 4 by 5 grid. The arena was a clean large cage (42 cm X 22 cm X 20 cm). Corncob bedding same as the home cage bedding was used to make a 4 centimeter thick layer on the bottom of the arena. The individual MB session lasted 60 minutes, during which the mouse was allowed free movement within the MB arena, but had no access to food or water. After 60 minutes the mouse was promptly returned to its group housing home cage, resuming *ad libitum* access to food and water. Lighting was similar to typical levels during the mouse's light cycle, and all testing was performed during the mouse's light cycle. This test was chosen after an initial screen of various behavioral tasks to determine intra-subject repeatability illustrated that marble burying could be used for a full dose-response curve (data not shown).

An individual mouse completed 8 MB sessions, once per week (Figure 1B). The first 4 sessions would with either escitalopram or fluoxetine, followed by a week of rest, then 4 weeks of the other SSRI. The drug order was randomized across the experimental cohort so that each week animals were in each of the four drug doses for both drugs. Escitalopram, as escitalopram oxalate, was dissolved in sterile 0.9% saline, to doses of 0, 1, 3.2, and 10 mg/kg (mg of the salt per kg of body weight, administered at a volume of 6.67 μ L/g). Fluoxetine, as fluoxetine HCl, was also dissolved in saline, to doses of 0, 1, 10, and 32 mg/kg (mg of the salt per kg of body weight, administered at a volume of 6.67 μ L/g). Each MB testing day the mouse was weighed for accurate dosing. Thirty minutes prior to the MB session the mouse would receive an intraperitoneal injection (i.p.) of the drug and dose for that session, and be returned to home cage. After 30 minutes the mouse would be placed in the MB arena for the 60 minute session. A marble was buried if more than half of it was buried. Studies included male and female hSERT-wt, hSERT-mut, and hSERT-wt/mut mice, 8–12 weeks old at the start of the experiment. There were 17 hSERT-wt, 26 hSERT-Emory and 22 hSERT-wt/mut mice that completed all 8 MB sessions.

Statistics were carried out using SPSS (IBM). A repeated-measures ANOVA was used to examine genotype X dose X drug effects for the knockin mice. Median effective dose (ED₅₀) values were determined and compared in Prism 6.0 (GraphPad). Individual mice were removed if they buried 0–2 or 18–20 marbles in the saline condition for either drug since these were two standard deviations from the mean number of marbles buried without drug on board. To account for inter-mouse variation in burying levels in the saline treated

condition, the knockin mouse data were normalized. The raw values of marbles buried per drug dose were normalized to that individual mouse's number of marbles buried in the saline condition. Because each mouse had two saline values, one per each block of four weeks of an individual drug, the number of marbles per drug was normalized to the saline week of that same block. This normalized data was then curve fit for analysis and determination of ED₅₀ values.

2.6 Tail Suspension Test

The TST is a standard screen of SSRI action, making it an optimal screen of the allosteric site for escitalopram on the hSERT. Mice were affixed to a horizontal bar with tape 30 cm from the ground. A tube of plastic 1 centimeter in length was put on the tail of each mouse to prevent tail climbing. Each TST session lasted 6 minutes and was filmed for later scoring. Within the 6 minute TST session duration immobile was scored using Stopwatch+ (Center for Behavioral Neuroscience, Emory University, Atlanta, GA). Immobility was defined as no movement of limbs or head. After the TST session the mouse was promptly returned to its group housing home cage, resuming *ad libitum* access to food and water. Lighting was similar to typical levels during the mouse's light cycle, and all testing was performed during the mouse's light cycle.

The TST assay followed a similar timeline to the MB assay (Figure 1B). Eight TST sessions were completed over 9 weeks, with each mouse completing 4 escitalopram oxalate doses (0–10 mg/kg, administered at a volume of 6.67 μ L/g) and 4 fluvoxamine maleate doses (0–32 mg/kg, administered at a volume of 6.67 μ L/g) in four weeks. A 2 week washout period occurred between compounds and the order of the drugs and doses was counterbalanced across the testing cohort. Escitalopram, as escitalopram oxalate, was dissolved in sterile 0.9 % saline, to doses of 0, 1, 3.2, and 10 mg/kg (mg of the salt per kg of body weight). Fluvoxamine does not interact with the allosteric site *in vitro* and was chosen after pilot data found no dose-response effect of fluoxetine in the TST but fluvoxamine did elicit a dose-response effect in the TST (data not shown). Pilot data also confirmed the ability to repeat the TST within the same mouse on a once per week basis with both compounds. Fluvoxamine, as fluvoxamine maleate, was also dissolved in saline, to doses of 0, 1, 10, and 32 mg/kg (mg of the salt per kg of body weight). Each TST day the mouse was weighed for accurate dosing. Thirty minutes prior to the TST session the mouse would receive an intraperitoneal injection (i.p.) of the drug and dose for that session, and be returned to home cage. After 30 minutes the mouse would be placed for the TST for the 6 minute session. Sessions were scored without knowledge of genotype, drug, or dose. Male and female hSERT-wt, hSERT-mut and hSERT-wt/mut mice, aged 8–12 weeks at the beginning of the experiment were used. There were 15 hSERT-wt, 11 hSERT-Emory and 21 hSERT-wt/mut mice that completed all 8 TST sessions.

Statistics were carried out using SPSS (IBM) as with MB. A repeated-measures ANOVA was used to examine genotype X dose X drug effects for the knockin mice. ED₅₀ values were determined and compared in Prism 6.0 (GraphPad). Mice that did not complete all 8 TST sessions were excluded from data analysis in the knockin mouse studies. Any mouse that was immobile for fewer than 60 seconds or longer than 300 seconds of the 6 minute

session was also excluded from analysis. To account for variation in the saline group and across the drug groups in the TST data, the data was normalized. The raw values of duration immobile per drug dose were plotted and fit to a curve. The unconstrained value for the top of this raw data fit curve was then used to normalize the raw data. This was done individually by genotype. The normalized data was then fit to a curve for further data analysis and determination of ED₅₀ values.

3. Results

3.1 Autoradiography

Upon visual inspection of the film it was obvious that the mSERT mice expressed the SERT protein more than any of the knockin lines. This qualitative analysis was supported by the quantitative analysis in the frontal cortex and the raphe nucleus, although there was no significant difference in the hippocampus (data discussed below, values in Figure 2A). Overall, this indicated that the knockin protein expresses less readily than the endogenous mSERT. Importantly, there were no significant differences in SERT expression between the hSERT-wt and hSERT-mut mice at any of the three measured regions, indicating that the two mouse lines are appropriate for comparison in identifying the role of the allosteric site.

In the frontal cortex there was a significant effect of genotype on SERT expression, as measured in nCi/mg, $F(3,24) = 4.251$, $p = 0.017$ (Figure 2B). Post hoc testing determined that in mSERT mice the SERT expressed significantly more than in hSERT-wt and hSERT-wt/mut mice ($p < 0.05$ for both), and trended towards increased expression compared to hSERT-mut mice ($p = 0.052$). There was no significant difference between the knockin lines.

In the hippocampus there was no significant difference in SERT expression across then genotypes, $F(3,24) = 0.927$, $p = 0.445$ (Figure 2C). The absolute value of nCi/mg measured in the mSERT is greater than any of the knockin lines, but this did not reach significance. Additionally, it is important to note that there is no difference between the knockin lines, as well.

In the raphe nucleus there was a significant effect of genotype on expression of the SERT, $F(3,24) = 4.615$, $p = 0.012$ (Figure 2D). Post hoc tests identified mSERT mice as expressing significantly more SERT compared to hSERT-wt and hSERT-wt/mut mice ($p < 0.05$ for both), and trended towards an increase over hSERT-mut mice ($p = 0.051$). There were no significant differences in expression between the knockin lines.

3.2 Microdialysis

The serotonin response to SSRI drug challenge was measured by comparing the AUC of the response in the two dialysate samples after the initiation of SSRI challenge. AUC was also calculated for the four baseline samples. Using a Student's *t*-test, baseline AUC values were compared to determine there is no genotype effect, $p = 0.500$ (Figure 3A). Uncorrected for probe recovery, baseline serotonin for hSERT-wt was $1.55 \text{ nM} \pm 0.74$ and for hSERT-mut was $1.97 \text{ nM} \pm 2.13$. A repeated-measures ANOVA comparing the AUC found no genotype X dose X drug interaction effect, $F(2,54) = 1.242$, $p = 0.297$ (Figure 3B). However, there

was a significant main effect of dose (drug challenge: baseline, low or high), $F(2,54) = 5.879$, $p = 0.005$. Pairwise comparisons revealed that both the low and high dose SSRI challenges evoked a significant increase in extracellular serotonin over baseline, $p = 0.013$ and 0.041 , respectively, indicative that the hSERT-wt and hSERT-mut present was functional and able to respond to the application of an SSRI. Surprisingly, and in contrast to pilot data, there was no difference between the low and high doses, $p = 0.693$. There was no dose X drug effect indicating that the response to escitalopram and fluoxetine was the same at each dose of challenge, $F(2,54) = 0.588$, $p = 0.559$. There was a genotype X dose effect, $F(2,54) = 4.027$, $p = 0.023$, but as there was no significant genotype main effect, $F(1,27) = 2.124$, $p = 0.157$, this interaction effect was likely driven by the large main effect of dose. While there appears to be a visual difference in the low dose of escitalopram response between the two genotypes, this was not confirmed by *post hoc* tests.

3.3 Marble burying

In the hSERT-wt and hSERT-mut lines, there was a significant genotype X dose X drug effect in MB, $F(6,186) = 2.516$, $p = 0.023$ (Figure 4A & 4B). It is important to note that pilot studies found no sex-based differences, thus male and female mice were kept in a single group. There was a significant main effect of dose, $F(3,186) = 67.676$, $p < 0.001$, indicative that the MB test in the knockin mice followed the expected response pattern. The escitalopram oxalate ED₅₀ for hSERT-wt was 2.528 mg/kg, hSERT-mut was 24.06 mg/kg, and hSERT-wt/mut was 14.41 mg/kg (Figure 4A inset). There was a significant effect of genotype on the escitalopram ED₅₀ values, $F(2,257) = 3.175$, $p = 0.044$. Post hoc tests revealed there was a significant difference between hSERT-wt and hSERT-mut ED₅₀, $p < 0.05$. The fluoxetine HCl ED₅₀ for hSERT-wt was 6.450 mg/kg, hSERT-mut was 12.23 mg/kg, and hSERT-wt/mut was 8.598 mg/kg (Figure 4B inset). There was no significant effect of genotype on the fluoxetine ED₅₀ values, $F(2,257) = 1.381$, $p = 0.253$.

These results from the knockin mice support the hypothesis that the allosteric site for escitalopram on the hSERT does have a behavioral role in the MB task following systemic escitalopram exposure. The significant increase in ED₅₀ in the absence of the allosteric site on the hSERT indicates a rightward shift in the dose-response curve for escitalopram. Consequently more escitalopram is necessary to achieve the same effect on the MB test in hSERT-mut mice compared to intact hSERT-wt mice. No significant change in ED₅₀ for allosterically-inactive fluoxetine between genotypes further bolsters the hypothesis that the observed escitalopram effect is due to the presence or absence of the allosteric site.

3.4 Tail Suspension Test

Pilot work did suggest repeated TST sessions would result in some learning of the task, and counterbalancing was meant to negate that effect; analysis of the hSERT lines did determine there was no difference in the saline group mice week to week, $F(7,51) = 1.806$, $p = 0.110$ (data not shown). Pilot data also noted no sex difference, thus male and female mice were kept in a single group for the below analysis.

In the knockin lines there was no significant genotype X dose X drug effect in duration immobile in the TST, $F(2,674, 44) = 0.454$, $p = 0.694$ (Greenhouse-Geisser correction;

Figure 4C & 4D). There was a significant main effect of dose in duration immobile in the TST, $F(1.308, 57.547) = 41.149$, $p < 0.001$ (Greenhouse-Geisser correction), indicative that the TST worked as expected. The ED₅₀ values were also examined for a genotype effect. The escitalopram oxalate ED₅₀ for hSERT-wt was 4.770 mg/kg, hSERT-mut was 8.205 mg/kg, and hSERT-wt/mut was 5.883 mg/kg (Figure 4C). There was no significant effect on genotype for the escitalopram oxalate ED₅₀ values, $F(2,182) = 0.731$, $p = 0.483$. The fluvoxamine maleate ED₅₀ for hSERT-wt was 10.11 mg/kg, hSERT-mut was 16.57 mg/kg, and hSERT-wt/mut was 13.16 mg/kg (Figure 4D). There was no significant effect of genotype on the fluvoxamine maleate ED₅₀ values, $F(2,182) = 0.693$, $p = 0.501$.

These data suggest that escitalopram's allosteric site on the hSERT has no behavioral role in the TST. There was no genotype effect on either duration immobile or latency to immobility, and there was no genotype effect on ED₅₀ for escitalopram in the TST. This does not support the hypothesis that the absence of the allosteric site would cause a shift in behavioral output in the TST. The main effect of dose for duration indicates that the TST was a successful test as suggested by the pilot work, however the presence or absence of the allosteric site does not alter the escitalopram effect in the TST.

4. Discussion

Overall, the data for these experiments suggest that the allosteric site is not of great importance to escitalopram's *in vivo* action. Autoradiography confirmed the expression of the hSERT knockin proteins. Microdialysis studies examined the physiological role of the allosteric site in the ventral hippocampus. MB and the TST studied the behavioral output of the allosteric site. Ideally these studies would have converged to an answer of what is the role of the escitalopram allosteric site on the hSERT. Yet these studies do not come to a robust conclusion, rather they highlight that, at most, the allosteric site has a fine tuning role in the pharmacology of escitalopram.

In characterizing the knockin mouse lines, autoradiography revealed that hSERT expression was reduced compared to that of mSERT in typical C57BL/6J mice. The reduced expression could have played into the microdialysis and behavioral results; however it does not seem that it was impactful. Further, a lack of difference in expression between the knockin lines maintains the ability of the two knockin lines to be compared to each other. While there may be some differences between the knockin lines and wildtype mice, it is ultimately the role of the knockin lines to be compared to each other to screen the role of the allosteric site.

Taken together, the microdialysis and behavioral data create an interesting picture of how the allosteric site on the hSERT plays a role in escitalopram's mechanism of action. The hypothesis was that removing the allosteric site on the hSERT would result in a blunting of escitalopram's effects so that a larger dose would be required to achieve the same level of effect as in hSERT-wt mice. The MB data supported this hypothesis, with the modest rightward shift in ED₅₀ values for escitalopram in the absence of the allosteric site. The microdialysis and TST data, however, do not support the hypothesis, and rather suggested no role of the allosteric site in escitalopram's effects in those studies. How these data fit

together to further characterize escitalopram's allosteric site on the hSERT will be discussed in more detail below.

The escitalopram allosteric site is hypothesized to produce an increase in extracellular serotonin. This would be in addition to the buildup of extracellular serotonin that results from inhibiting the transporter via the primary binding site. Because the transporter is successfully inhibited via primary binding, the additional amount of serotonin should not be of a very large magnitude. Consequently this small amount of extra serotonin is likely only capable of making a fine effect or is only effective in junctures very sensitive to serotonin levels. In light of this, MB may be sensitive to fine tuning of serotonin, whereas TST may not be, allowing for the measured pattern of escitalopram effects. Had the microdialysis experiments revealed in the ventral hippocampus there is a difference in extracellular serotonin levels between the hSERT-wt and hSERT-mut mice after escitalopram challenge this may have explained the MB result. However, there was no difference in microdialysis. Instead this suggests that the MB effect is not entirely hippocampal-linked.

Another possibility is that the microdialysis experiments were unable to detect differences between the hSERT-wt and hSERT-mut mice. There was variation in the mouse-to-mouse level of response to SSRI application. For example, while there was no difference in the baseline levels of serotonin between groups, a baseline value of 1 nM extracellular serotonin followed by a value of 10 nM extracellular serotonin after escitalopram challenge results in a 1000% change from baseline. On the other hand, starting at 2 nM extracellular serotonin would result in only a 500% change from baseline. A fine effect of genotype could have potentially been lost in the data analysis. The ventral hippocampus was chosen for study due to its abundance of serotonin. Perhaps another region less saturated in serotonin would provide a measurable view of effects caused by the presence or absence of the allosteric site. Although speculative, perhaps one of those regions is sensitive to the allosteric site and is a region driving the separation of the genotypes in the MB test.

What is interesting about the MB data is it does seem to be in line with clinical work which identified escitalopram to have a significant but modest superiority to citalopram (Montgomery *et al.*, 2001, 2007; Gorman *et al.*, 2002; Waugh and Goa, 2003; Lepola *et al.*, 2004; Moore *et al.*, 2005; Cipriani *et al.*, 2009; Ali and Lam, 2011). In clinical studies and meta-analyses of these studies the data shows that patients respond more quickly to escitalopram than to citalopram, and faster than placebo and other antidepressants. Yet the separation between escitalopram and other compounds does not have a large magnitude and patients still respond to the other compounds. The MB effect due to the presence or absence of the allosteric site is measured as an acute response in mice and is clearly not an exact model of any particular psychiatric disorder, rather a screen of compounds that may relieve psychiatric symptoms (e.g. SERT antagonism). Indeed, the reader should consider our use of SSRIs in these studies as tools to probe serotonin transporter function and not as being means to understanding antidepressant pharmacology. Perhaps MB in mice with acute drug challenge is sensitive to correlates of faster response to SSRIs in humans. And perhaps the TST after acute drug challenge is only a screen of if a compound is able to evoke a response in humans. While this is purely speculative, it would be an explanation for why there was a different conclusion for the two behavioral tests.

Together the physiological and behavioral data suggest that there is a modest effect of the allosteric site in escitalopram action, depending upon the output examined, but it is not a robust effect that supports further pursuing of the site as a specific target of pharmacological merit *in vivo*. While the MB finding on its own is interesting, the lack of corroboration from another behavioral screen of SSRI activity and microdialysis suggests that the MB finding does not measure an important global effect. A finding that supported even a modest effect of the absence of the allosteric site in escitalopram's activity in all of the physiological and behavioral data would have lent itself to further study, including manipulations to the mice to model depression to examine how the allosteric site affects escitalopram's effects in a depressed state.

This conclusion seems surprising in light of the hypotheses based on *in vitro* and *in vivo* work previously completed. Yet when the concentration of escitalopram in human serum and CSF are compared to the concentrations used to observe allosteric activity *in vitro*, there are some obvious reasons why the studies herein may not replicate the prior work. The reported concentration range for escitalopram in human serum and CSF is between 30 and 100 nM (Sidhu *et al.*, 1997; Zhong *et al.*, 2011). These nanomolar concentrations are in contrast to the micromolar concentrations necessary to observe allosteric activity *in vitro* (Chen, Larsen, Neubauer, *et al.*, 2005). The escitalopram doses used in the MB and TST studies are more clinically relevant, and the concentrations administered in the microdialysis studies (0.0462 μ M and 0.1 μ M) are in the observed *in vivo* range. Perhaps at a higher dose or concentration the hSERT-wt and hSERT-mut mice would have revealed an *in vivo* allosteric effect for escitalopram, but that would ultimately not be clinically relevant. Therefore the same conclusion is met, that the allosteric site does not have a robust or valuable *in vivo* effect. With careful attention to clinically relevant dosing, studies using a similar allosteric knockin mouse also concluded that the role of the allosteric site failed to reach significance (Jacobsen *et al.*, 2014).

5. Conclusion

These studies of the characterization of the hSERT-wt and hSERT-mut mice and the subsequent comparison of the knockin lines to examine the *in vivo* role of the escitalopram allosteric site on the hSERT came to the conclusion that the allosteric site does not present a robust *in vivo* role. The knockin lines themselves remain a valuable asset to examine any future questions about the allosteric site, and the hSERT-wt mice alone can be of use to examine and manipulate the hSERT protein in an experimental system.

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Highlights

- Novel insertion of the human SERT and an allosteric site null human SERT
- No effect of the absence of allosteric site in microdialysis or TST
- Minor escitalopram ED₅₀ rightward shift in absence of allosteric site in MB
- Overall, no significant role for the allosteric site in escitalopram activity

Figure 1.

Experimental Timelines. A. Timeline of microdialysis experiment. Sample collected every 20 minutes. Time = 0 min at when baseline collection begins after 80 minute recovery period. The thick bar above the timeline indicates time of drug exposure. Probe was removed from the mouse at time of euthanasia. Serotonin recovery was performed before and after the probe was in the mouse. The time of drug exposure matched with the first sample for the drug challenge. B. For MB and TST each mouse had 8 sessions over 9 weeks. Each mouse had the 4 doses of escitalopram oxalate (0–10 mg/kg) or 4 doses of control SSRI (0–32 mg/kg fluoxetine HCl for MB or 0–32 mg/kg fluvoxamine maleate for TST) over the first 4 weeks, followed by 2 week washout period, then 4 weeks using the other SSRI. Per individual session the drug was administered 30 minutes prior to the test via i.p. injection.

Figure 2.

Knockin hSERT expression in select brain regions of the mouse. A. Mean $\pm$ SEM values for autoradiography. B. Cortex means per genotype. C. Hippocampus per genotype. D. Raphe per genotype.

Figure 3.

Serotonin measured in microdialysate before and after escitalopram challenge. Mean $\pm$ SEM AUC of measured serotonin in ventral hippocampus. AUC = 0 would indicate no serotonin present. A. Baseline serotonin is not different between the genotypes. B. Response to low (0.0462 μ M) and high (0.1 μ M) escitalopram or fluoxetine challenge. Significant response over baseline (shown in 3A) but no significant effect between genotypes.

Figure 4.

Behavioral response to escitalopram challenge. Mean $\pm$ SEM response to escitalopram or control in MB and TST. A. MB response to escitalopram, inset shows significant increase of ED₅₀ upon absence of allosteric site. B. MB response to fluoxetine, inset shows no genotype effect on ED₅₀. C. TST response to escitalopram, inset shows no genotype effect on ED₅₀. D. TST response to fluvoxamine, inset shows no genotype effect on ED₅₀. Grey bar indicates variance in saline condition.