Pharmacology, Biochemistry and Behavior 141 (2016) 50–57

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

# *In vivo* investigation of escitalopram's allosteric site on the serotonin transporter

Karen E. Murray [a,1], Kerry J. Ressler [b,1], Michael J. Owens [a,*]

[a] Laboratory of Neuropsychopharmacology, 101 Woodruff Circle, Suite 4000, Department of Psychiatry and Behavioral Sciences, Emory University, Atlanta, GA 30322, United States
[b] Laboratory of Molecular Neurobiology of Fear, 101 Woodruff Circle, Suite 4000, Department of Psychiatry and Behavioral Sciences, Emory University, Atlanta, GA 30322, United States

ARTICLE INFO

*Article history:*
Received 3 October 2015
Received in revised form 20 November 2015
Accepted 23 November 2015
Available online 24 November 2015

*Keywords:*
Serotonin transporter
Allosteric
SSRI
Marble burying
Tail suspension test
Escitalopram

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.



## 1. Introduction

Selective serotonin reuptake inhibitors (SSRIs[2]) 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 et al., 2005a; 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

* Corresponding author at: 101 Woodruff Circle, Suite 4000, Department of Psychiatry and Behavioral Sciences, Emory University, Atlanta, GA 30322, United States.
*E-mail address:* mowens@emory.edu (M.J. Owens).

[1] KEM has since moved to Children's Healthcare of Atlanta, Atlanta, GA, USA; KJR has since moved to Mclean Hospital, Affiliate of Harvard Medical School, Belmont, MA, USA.

[2] Abbreviations: AUC, area under the curve; $ED_{50}$, 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.

http://dx.doi.org/10.1016/j.pbb.2015.11.010
0091-3057/

*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 et al., 2005b).

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 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 that 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 h 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 autoradiography and referred to as mSERT mice. All experiments were conducted in the animals' 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.

[3] The 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

### 2.2. Drugs

Escitalopram oxalate was a generous gift from H. Lundbeck USA (Paramus, NJ). [$^3$H]-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 °C until use. Brains were sliced at −16 °C on a cryostat at 20 μm 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' *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 °C until use.

The radioligand for the autoradiography was [$^3$H]-citalopram. Brain slices were thawed to room temperature then preincubated in SERT buffer (52.2 mM Tris HCl, 126.4 mM NaCl, 5.26 nM KCl) for 15 min. Total binding and non-specific binding solutions were also made in SERT buffer. The total binding solution was 2 nM [$^3$H]-citalopram, and the non-specific binding solution was 2 nM [$^3$H]-citalopram with 1 μM non-radiolabled escitalopram. The slides incubated in either the total binding or non-specific binding solutions for 60 min. The slides were then washed 2 times for 10 min each in 0 °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.