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The interaction of escitalopram and R-citalopram at the *human* serotonin transporter investigated in the mouse

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Abstract

Rationale—Escitalopram is a superior antidepressant to racemic citalopram. It has been hypothesized that binding of R-citalopram to the serotonin transporter (SERT) antagonizes escitalopram binding to and inhibition of the SERT, curtailing the elevation of extracellular 5-hydroxytryptamine (5-HT_{Ext}), and antidepressant efficacy. Further, it has been suggested that a putative *allosteric* binding site is important for binding of escitalopram to the primary, *orthosteric*, site, and for R-citalopram's inhibition hereof.

Objectives—*Primary*: Investigate at the *human* (h)SERT, at clinical relevant doses, whether R-citalopram antagonizes escitalopram-induced 5-HT_{Ext} elevation. *Secondary*: Investigate whether abolishing the putative allosteric site affects escitalopram-induced 5-HT_{Ext} elevation and/or modulates the effect of R-citalopram.

Methods—Recombinant technology; *in vivo* microdialysis; receptor binding; pharmacokinetics; 5-HT sensitive behaviors (tail suspension, marble burying).

Results—We generated mice expressing either the wild-type human SERT (hSERT^{WT}) or hSERT carrying amino acid substitutions (A505V, L506F, I507L, S574T and I575T) collectively abolishing the putative allosteric site (hSERT^{ALI/VFL+SI/TT}). One mg/kg escitalopram yielded clinical relevant plasma levels and brain levels consistent with therapeutic SERT occupancy. Importantly, escitalopram-induced 5-HT_{Ext} elevation was *not* decreased by R-citalopram co-treatment. Further, escitalopram-induced 5-HT_{Ext} elevation was not affected by loss of the

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allosteric site. The behavioral effects of the clinically relevant escitalopram dose were small, tending to be enhanced by R-citalopram co-administration.

Conclusions—We find no evidence that R-citalopram directly antagonizes escitalopram or that the putative allosteric site is important for hSERT inhibition by escitalopram. Our findings points to mechanisms for R-citalopram antagonism of escitalopram's antidepressant action other than direct antagonistic binding interactions at the hSERT.

Keywords

5-HT; allosteric; antidepressant; escitalopram; R-citalopram; microdialysis

Introduction

Current antidepressants bring remission to only 40 % of patients (Gibbons et al. 2012). Regrettably, once promising compounds, such as neuropeptide antagonists (Binneman et al. 2008; Griebel et al. 2012; Keller et al. 2006; Manning et al. 2012; Neurocrine 2013) and selective noradrenaline reuptake inhibitors (Eyding et al. 2010; Michelson et al. 2007; Preti 2002) appear to have failed as antidepressants. Today, enhancing extracellular ($_{Ext}$) 5-hydroxytryptamine (5-HT, serotonin), mainly by selective serotonin-reuptake inhibitors (SSRIs), remains the primary antidepressant principle (Jacobsen et al. 2012a), although targeting the NMDA receptor appears to represent one promising avenue for a truly novel antidepressant (Aan Het Rot et al. 2012). The SSRI citalopram consists of a 1:1 ratio of S-citalopram (a.k.a. escitalopram) and R-citalopram (Sanchez 2006). In humans, slower metabolism results in at least two-fold (in some patients up to four-fold) higher plasma levels of R-citalopram vs. escitalopram after chronic treatment (Brosen and Naranjo 2001; Sidhu et al. 1997). Escitalopram has a ~50 fold higher affinity for the human serotonin-reuptake transporter (hSERT) compared to R-citalopram (Chen et al. 2005; Sanchez 2006; Zhong et al. 2009). Remarkably, R-citalopram appears to antagonize the antidepressant effect of escitalopram. Head-to-head double-blind clinical trials, as well as meta-analyses, have repeatedly demonstrated the antidepressant superiority of escitalopram vs. twice dose citalopram (Colonna et al. 2005; Kennedy et al. 2006; Lepola et al. 2004; Lepola et al. 2003; Llorca et al. 2005; Moore et al. 2005; Yevtushenko et al. 2007). By 2009 escitalopram was the most prescribed antidepressant in the US (PsychCentral 2009). The molecular pharmacology of R-citalopram's antagonism of escitalopram remains to be defined. Its definition could inform on the mechanism of antidepressant action and potentially aid the development of improved therapies.

R-citalopram antagonizes escitalopram in a variety of depression-relevant animal models, e.g. the rat chronic mild stress paradigm (Sanchez et al. 2003b) and the mouse 5-hydroxytryptophan-induced behavioral syndrome (Sanchez and Kreilgaard 2004). In rats, acute administration of R-citalopram in a 2:1 or 4:1 ratio antagonized the 5-HT $_{Ext}$ -enhancing effect of escitalopram, as assessed by microdialysis (Mork et al. 2003), a finding essentially replicated by others (Mansari et al. 2007). Further, in rats acute R-citalopram antagonized the suppression of dorsal raphe 5-HT neuronal firing induced by acute escitalopram administration *in vivo* (Mansari et al. 2007). The mechanism underlying these effects of R-citalopram has been suggested to involve antagonism of escitalopram binding to

the SERT by R-citalopram (Sanchez 2006; Zhong et al. 2011), although direct evidence is limited. Further, whether R-citalopram antagonizes escitalopram at *hSERT* has so far not been possible to address. This is important. Generalizing pharmacology between species can be precarious. Considerable species differences in ligand pharmacology have been reported for a range of important drug targets despite high amino acid homology (Cristalli et al. 2008; Gitter et al. 1991; Ireland-Denny et al. 2001; Iyengar et al. 1997; Leffler et al. 2009). Indeed, approximately five percent of the amino acid sequence differs between *hSERT* and rodent SERTs (Blakely et al. 1991; Chang et al. 1996). Substantial species differences in SERT affinities for tricyclic and SSRI antidepressants exist (Barker et al. 1994; Zhong et al. 2009). Further, a single amino acid substitution abolishes escitalopram binding at the orthosteric site on the *mSERT* (Thompson et al. 2011), illustrating the potentially high functional impact of even small sequence differences.

In *humans*, a positron emission tomography (PET) study found that acute therapeutic doses of 10 mg escitalopram caused significantly *lower* *hSERT* occupancy compared to 10 mg citalopram (69% vs. 78 %). These human data suggests that R-citalopram binds additively to the *hSERT* rather than antagonizing escitalopram binding, as suggested by animal data, and at a magnitude commensurate with the lower affinity of R-citalopram for *hSERT* (Lundberg et al. 2007). Similarly, a single-photon emission computed tomography (SPECT) study also found no evidence of inhibition of escitalopram binding to the *hSERT* by R-citalopram after acute dosing, although in this study the difference between escitalopram (10 mg) and citalopram (20 mg) occupancy (64 % vs. 70 %) did not reach significance (Klein et al. 2006). However, the latter investigators also reported that *hSERT* occupancy at steady state (10 days treatment) was significantly higher with 10 mg escitalopram (82 %) than with 20 mg citalopram (64%) (Klein et al. 2007). Thus, in contrast to most animal data, available human data are in fact more consistent with a R-citalopram inhibition of escitalopram mechanism only becoming apparent upon chronic treatment.

An influential hypothesis posits that R-citalopram counteracts escitalopram's binding to the high-affinity orthosteric site on SERT via interactions at a second, spatially distinct, low-affinity *allosteric* site (Plenge et al. 2007; Sanchez 2006; Zhong et al. 2011). A central pillar of this hypothesis is the observation that an excess of unlabeled escitalopram *inhibits* – rather than accelerates – the dissociation of ³H-labeled escitalopram from SERT (Plenge et al. 2007; Zhong et al. 2011; Zhong et al. 2009). According to this hypothesis, binding of one escitalopram molecule to the allosteric site allows a second escitalopram molecule to bind more tightly to the orthosteric site, thereby causing a more complete inhibition of the SERT and better/faster antidepressant action (Sanchez 2006; Zhong et al. 2011). Enhanced SERT blockade could have clinical significance since steady state clinical SSRI occupancy of SERT appears to plateau at 85–90%, even with high systemic levels (Meyer et al. 2004). Escitalopram's retardation of its own dissociation from SERT can readily be demonstrated *in vitro*. However, mid- to high μ M concentrations are required (Plenge et al. 2007; Zhong et al. 2009). In contrast, clinical steady state cerebrospinal fluid and plasma concentrations are in the mid nM range (Nikisch et al. 2005), 1000 fold lower. This apparent incongruence is difficult to reconcile, although escitalopram accumulates four-fold in brain *vs.* plasma in rats (Kreilgaard et al. 2008). Another complexity is that R-citalopram in fact also retards the

dissociation of escitalopram from SERT, albeit less potently (Chen et al. 2005; Plenge et al. 2007). Thus, while the allosteric hypothesis is heuristically attractive, significant discordance exists within the supporting data. Further, preferably *in vivo*, studies are therefore necessary.

Pre-clinical pharmacological investigations should ideally employ clinically relevant dosing regimens. Sub-clinical doses may elicit an insufficient pharmacodynamic response, while supra-clinical clinical doses can have off-target effects. For instance, despite a 10^4 fold selectivity for SERT over the noradrenaline transporter (Owens et al. 2001), an escitalopram dose (8 mg/kg) yielding plasma levels >20 fold above clinical levels increased extracellular noradrenaline in SERT knockout mice, seemingly by blocking the noradrenaline transporter (Nguyen et al. 2012).

In the present study, we addressed the dilemma that some human pharmacology hypotheses can conceptually only convincingly be tested at the human drug target, but methodologically only tested in animals. We generated *humanized* mice with the gene for hSERT knocked into the mSERT gene locus. This enabled us to directly address our *primary objective*, namely the functional interactions of R-citalopram and escitalopram at the hSERT, using *in vivo* microdialysis, pharmacokinetics and behavioral paradigms, supported by *ex vivo* ligand binding approaches. To address our *secondary objective*, the role of the escitalopram putative allosteric site *in vivo*, we generated mice with a knockin of a mutated hSERT lacking the allosteric site. We subjected these mice to an identical analysis. Based on previous *in vitro* and *in vivo* data (reviewed in (Zhong et al. 2011)), our hypothesis was that es- and R-citalopram interactions, and a putative role of an allosteric site, involved immediate binding interactions. Hence, an acute, single-dose paradigm would be most appropriate and was employed here. Further, since the es- and R-citalopram interactions are believed to occur specifically at the drug concentrations encountered in a clinical setting (Sanchez 2006), we ascertained that the escitalopram dose employed produced clinically relevant plasma escitalopram concentrations.

METHODS AND MATERIALS

Mice

Mice carrying hSERT wild type ($hSERT^{WT}$) or hSERT with five amino acids substitutions (A505V, L506F, I507L, S574T and I575T; $hSERT^{ALI/VFL+SI/TT}$) were generated by homologous recombination (see Supplementary Methods). These substitutions collectively abolish escitalopram's allosteric effect *in vitro* (Neubauer et al. 2006; Zhong et al. 2009). hSERT mice were maintained on a mixed 129S6/SvEv $\times$ C57BL/6j background and bred via heterozygous $hSERT^{WT-ALI/VFL+SI/TT} \times hSERT^{WT-ALI/VFL+SI/TT}$ crosses. The presence or absence of hSERT and mSERT mRNA transcripts was determined using restriction enzyme analysis of amplified cDNA (see Supplementary Methods). Standard ($mSERT^{WT}$) mixed 129S6/SvEv $\times$ C57BL/6j background mice were bred in-house (Duke University). Littermate mice were housed 3–5 per cage with food and water available *ad libitum* on a 12 h light-dark cycle at an ambient temperature of 21 ± 2 °C. Experiments were conducted during the light phase in age- and gender-matched littermate mice (3–6 months old). Experiments were conducted in accordance with the National Institutes of Health guidelines

for the care and use of animals and an approved animal protocol from the Duke University Animal Care and Use Committee.

Drugs

Escitalopram oxalate, R-citalopram oxalate and ^3H -escitalopram were obtained from H. Lundbeck A/S (Valby, Denmark). Enantiomeric purity was verified to be >99.8 % (Hans Petersen, Lundbeck, personal communication, data not shown). All drug combinations were administered in sterile saline as a single injection (I.P., 10 ml/kg).

Autoradiography

SERT autoradiography for gross anatomical distribution was conducted as previously (Pehrson et al. 2012), with modifications (see Supplementary Methods). In brief, SERT in slide-mounted tissue section were labeled with ^3H -escitalopram and imaged with a beta-imager. To account for the escitalopram affinity differences between the mouse genotypes, the concentration of ^3H -escitalopram was set at approximately K_D (1 nM in mSERT^{WT}, 2.3 nM in hSERT^{WT}, and 5.3 nM in hSERT^{ALI/VFL+SI/TT} mice).

^3H -escitalopram Saturation Binding

^3H -escitalopram binding was measured in brain membrane preparations (see Supplementary Methods) at increasing concentrations of ^3H -escitalopram from 0.1 nM to 12.8 nM and B_{Max} and K_D were calculated from the saturation binding curves as previously described (Plenge et al. 1990).

^3H -escitalopram Dissociation Binding

Dissociation rates were determined as previously described (Plenge et al. 2007) (and see Supplementary Methods). In brief, ^3H -escitalopram was added to the brain membranes (see Supplementary Methods) and incubated for 30 min at 0 °C in binding buffer. Dissociation was initiated by diluting samples in binding buffer containing escitalopram and/or R-citalopram in the indicated concentrations. ^3H -escitalopram remaining bound was assessed from 5 to 70 min.

Escitalopram Pharmacokinetics

Escitalopram 0.25, 0.5, 1 and 2 mg/kg were administered to mice of the background strain (mSERT^{WT} mice), brains and plasma were collected at 30, 60 and 90 min, stored at -80 °C and analyzed by mass spectrometry (see Supplementary Methods).

Frontal Cortex 5-HT_{Ext}

Microdialysis for 5-HT_{Ext} was performed as previously described (Jacobsen et al. 2012b) but using probes from BrainLink (Groningen, NL). In brief, a guide cannula (NM 040, BrainLink, Groningen, NL) was stereotactically implanted into the frontal cortex using coordinates (AP: 2.3mm, ML: 0.3mm, DV: 0.7mm) according to the atlas by Paxinos & Franklin (Franklin and Paxinos 1997). Following 48–96 h recovery the microdialysis probe (NO-NM-PAN 060-020) was inserted into the guide cannula approximately 16 h before sample collection. Then three 30 min dialysates were collected, where after drugs were

rapidly injected and six additional dialysates were collected. Dialysates were stored at -80°C and analyzed off-line by HPLC with electrochemical detection using standard methods, as described (Jacobsen et al. 2012b).

Frontal Cortex Tissue 5-HT

5-HT and 5-hydroxyindoleacetic acid (5-HIAA) levels were quantified as described (Jacobsen et al. 2012b). In brief, mice were rapidly decapitated, frontal cortex tissue was collected and homogenized by sonication, debris and membranes were pelleted by centrifugation, and 5-HT and 5-HIAA levels were quantified in the supernatant using HPLC (Jacobsen et al. 2012b).

5-HT_{Ext} sensitive behaviors

Marble burying and the *tail suspension test* were included here because these behaviors are commonly sensitive to manipulations of brain 5-HT_{Ext} levels (Cryan et al. 2005; Hirano et al. 2005; Jacobsen et al. 2012b; Kobayashi et al. 2008; Njung'e and Handley 1991; Steru et al. 1985). These assay were included purely as ancillary, functional output measure of 5-HT_{Ext} alterations, not as indicators of “antidepressant-like” action.

Marble burying was assessed essentially as described (Jacobsen et al. 2012b). In brief, each mouse was injected with drugs or saline and 30 min later placed in a 30×40 cm rat cage containing a 10 cm layer of lightly compressed aspen shavings whereupon 24 red glass marbles were latticed. Sixty minutes later the mouse was removed and the number of visible marbles quantified from photographs by a blinded experimenter.

Tail suspension was performed essentially as described (Beaulieu et al. 2008). In brief, the mouse was injected with drugs or saline and 30 min later suspended by its tail using adhesive tape in an automated tail suspension apparatus (gain = 4, threshold = 4; MedAssociates, Georgia, VT). Immobility was recorded over 6 min.

Statistics

Data were analyzed using GraphPad Prism version 5.0 for Mac OS 10 (GraphPad Software, San Diego California USA, www.graphpad.com). T-test, one-way ANOVA, two-way ANOVA or repeated measures (RM)-ANOVAs was used as appropriate. Significant ANOVAs were followed by Bonferroni's or Dunnett's post-hoc tests, as appropriate. $P < 0.05$ was considered significant. Data are presented as means $\pm$ standard error of mean (SEM).

RESULTS

Generation and characterization of hSERT mice

hSERT^{WT} and hSERT^{ALI/VFL+SI/TT} mice were generated by homologous recombination resulting in replacement of mSERT with either hSERT^{WT} or hSERT^{ALI/VFL+SI/TT}. Restriction enzyme analysis of cDNA confirmed the presence of hSERT mRNA in hSERT^{WT} and hSERT^{ALI/VFL+SI/TT} mice, but not in mSERT mice (Figure 1A). Conversely, mSERT mRNA was only detected in mSERT^{WT} mice (Figure 1B). While the protocol

employed here will not provide high resolution images, ^3H -escitalopram autoradiography indicated that the brain regional expression patterns of hSERT^{WT} and hSERT^{ALI/VFL+SI/TT} were overall similar to that of the native mSERT (Figure 1C), indication that hSERT expression is under control of the mSERT promoter. Given the differing escitalopram affinity for mSERT^{WT}, hSERT^{WT} and hSERT^{ALI/VFL+SI/TT}, the autoradiographic signal differences apparent between the three genotypes does not indicate differences in SERT protein expression. In fact, a quantitative approach, ^3H -escitalopram saturation binding in brain membranes, revealed that both hSERT proteins were expressed at similar levels (and ~50 % of the levels observed in mSERT mice) as deduced from computed maximal binding (B_{Max}) (Figure 1D). The affinities (K_d) of ^3H -escitalopram for the orthosteric site were determined to be 1.13 ± 0.228 , 2.25 ± 0.30 and 5.45 ± 0.59 nM for mSERT, hSERT^{WT} and hSERT^{ALI/VFL+SI/TT}, respectively (frontal cortex, Figure 1D), values agreeing with previous reports (Sur et al. 1997; Zhong et al. 2009). Frontal cortex tissue levels of 5-HT and 5-HIAA were identical between hSERT^{WT} and hSERT^{ALI/VFL+SI/TT} mice, and comparable to levels previously reported for mice of the background strain (Jacobsen et al. 2012b), indicating similar 5-HT synthesis and metabolism for mSERT, hSERT^{WT} and hSERT^{ALI/VFL+SI/TT} mice (Figure 1E). Thus, both hSERT genotypes express similar levels of hSERT, with the native expression pattern and with no confounding co-expression of mSERT. The moderately decreased affinity of escitalopram for the orthosteric binding site, concomitant to the loss of affinity for the allosteric site (see below), on hSERT^{ALI/VFL+SI/TT} was anticipated (Zhong et al. 2009). These alterations must be taken into consideration when interpreting the outcomes of the functional analysis with regard to our secondary objective, the role of the putative allosteric site, as will be discussed below.

Escitalopram and R-citalopram allosteric interactions at hSERT^{WT} and hSERT^{ALI/VFL+SI/TT} *ex vivo*

We wanted to ascertain that previously reported key allosteric phenomena at hSERT in cell culture *in vitro* (Neubauer et al. 2006; Plenge et al. 2007; Zhong et al. 2009) could be detected in tissue preparations from hSERT^{WT} mice *ex vivo*, and that these phenomena were abolished in hSERT^{ALI/VFL+SI/TT} tissues. When tested in brain membranes prepared from hSERT^{WT} mice, escitalopram dose-dependently retarded the dissociation of ^3H -escitalopram from hSERT (Figure 2A and C). The minimal effective concentration was $3.7 \mu\text{M}$ (Figure 2C). The highest concentration of escitalopram, $100 \mu\text{M}$, caused a 20-fold increase in the dissociation half-life of ^3H -escitalopram (Figure 2C). Similar results have been reported from cell lines transfected with hSERT (Neubauer et al. 2006; Plenge et al. 2007; Zhong et al. 2009). In membranes prepared from hSERT^{ALI/VFL+SI/TT} mice, escitalopram had no effect on the dissociation half-life of ^3H -escitalopram (Figure 2B and D), paralleling previous *in vitro* data (Zhong et al. 2009). Thus, we demonstrate that the previously reported allosteric phenomenon at the hSERT *in vitro* is present *ex vivo* in membranes from hSERT^{WT} mice, and that this allosteric effect is abolished in membranes from hSERT^{ALI/VFL+SI/TT} mice. Additionally, R-citalopram additively increased the dissociation half-life of ^3H -escitalopram in hSERT^{WT} membranes when combined with a low, $3 \mu\text{M}$, concentration of escitalopram (Figure 2E–F), again closely paralleling previous *in vitro* data (Plenge et al. 2007).

Pharmacokinetic analysis establishing a clinically relevant escitalopram dose

We administered 0.25, 0.5, 1 or 2 mg/kg escitalopram to mice of the background strain and assessed levels in plasma and brain at 30, 60 and 90 min. One mg/kg escitalopram produced plasma levels in the mid-clinical range (20–60 ng/ml) (Nikisch et al. 2005; Rao 2007) within the time span assessed (0–180min), while 0.5 mg/kg produced levels in the low clinical range (Figure 3A). The dose-response relationship was non-linear, with the 2 mg/kg dose yielding 4.4 fold higher maximal plasma escitalopram concentrations (C_{max}) than 1 mg/kg (189 vs. 43 ng/ml, respectively; Figure 3B). Based on published data (Kreilgaard et al. 2008), these brain escitalopram levels are consistent with a SERT occupancy of 75–90 % with the time interval studied (0 – 180min). As expected, brain levels of escitalopram were 4–8 fold higher than in plasma (Figure 3C) and clearance was slower, as manifested by rising brain:plasma ratios over time (Figure 3D). The brain:plasma ratio was unaffected by drug dose, except for the lowest, 0.25 mg/kg, dose. Thus, we chose 1 mg/kg escitalopram as the dose best simulating the levels occurring at the hSERT in a clinical scenario.

Effect of R-citalopram on the escitalopram 5-HT_{Ext}-enhancing action at the hSERT *in vivo* and the consequences of eliminating the allosteric site

To directly test *in vivo* the hypothesis that R-citalopram inhibits the 5-HT_{Ext}-enhancing action of escitalopram at hSERT (Sanchez 2006; Zhong et al. 2011) we performed *in vivo* microdialysis in hSERT^{WT} mice. The role of the putative allosteric site was assessed by parallel analysis of hSERT^{ALI/VFL+SI/TT} mice. Baseline levels of 5-HT_{Ext} were identical between hSERT^{WT} (6.63 ± 0.68 fmol/sample, n = 24) and hSERT^{ALI/VFL+SI/TT} (6.41 ± 0.48 fmol/sample, n = 24) mice, and only slightly and non-statistically (p > 0.1, t-test) elevated above levels observed in mSERT^{WT} mice (5.37 ± 0.51 fmol/sample, n = 23). This indicates that the hSERTs are functional and that the ALI/VFL+SI/TT mutations do not affect baseline hSERT function. Saline injections did not affect 5-HT_{Ext} levels in either genotype (one-way RM-ANOVA, p > 0.1; Figure 4C–D).

First, we administered the optimal 1 mg/kg escitalopram dose to hSERT^{WT} and hSERT^{ALI/VFL+SI/TT} mice. 5-HT_{Ext} increased by ~ 250%, further confirming the functionality of the hSERTs when expressed in mice. Importantly, the 5-HT_{Ext} response was identical between hSERT^{WT} and hSERT^{ALI/VFL+SI/TT} mice (Figure 4A). A threshold clinical range escitalopram dose, 0.5 mg/kg, did reveal a slightly, yet significantly lower 5-HT_{Ext} response in the hSERT^{ALI/VFL+SI/TT} mice (two-way RM-ANOVA, genotype × time interaction, p < 0.001, $F_{[9,208]} = 2.63$). However, this escitalopram dose is likely to yield SERT occupancy on the ascending limb of the occupancy curve (Kreilgaard et al. 2008). Under those conditions the moderate decrease in affinity for escitalopram at the orthosteric site on hSERT^{ALI/VFL+SI/TT} could become functionally significant, providing a simple explanation for the reduced 5-HT_{Ext} response in the hSERT^{ALI/VFL+SI/TT} mice to 0.5 mg/kg escitalopram. Thus, the allosteric site did not appear important for escitalopram's action at hSERT *in vivo* at clinical escitalopram concentrations.

In humans chronically treated with citalopram, R-citalopram systemic concentrations are at least double those of escitalopram (Brosten and Naranjo 2001; Sidhu et al. 1997). To determine whether R-citalopram antagonized the 5-HT_{Ext} enhancing effect of escitalopram

at the hSERT^{WT} we co-administered 2 mg/kg R-citalopram with 1 mg/kg escitalopram. In contrast to *in vivo* reports on rSERT (Mansari et al. 2007; Mork et al. 2003), R-citalopram did not antagonize escitalopram's 5-HT_{Ext}-enhancing effect at hSERT^{WT} (Figure 4C). In fact, R-citalopram add-on appeared to weakly *augment* escitalopram's action, although significantly only in hSERT^{ALI/VFL+SI/TT} mice (two-way RM-ANOVA, treatment $\times$ time interaction, $p = 0.0125$, $F_{[8,136]} = 2.56$), the latter likely reflecting that R-citalopram has slightly (i.e. two-fold) increased affinity for hSERT^{ALI/VFL+SI/TT} compared to hSERT^{WT} (Zhong et al. 2009). Thus, we found no evidence that R-citalopram antagonizes the action of escitalopram under the present experimental conditions.

R-citalopram alone at 2 mg/kg increased 5-HT_{Ext} in hSERT^{ALI/VFL+SI/TT} mice only (two-way RM-ANOVA; effect of time: $p < 0.001$, $F_{[8,88]} = 9.29$; time $\times$ treatment: $p < 0.001$, $F_{[8,88]} = 7.41$; Figure 4E–F). Increasing the R-citalopram dose to 4 mg/kg caused significant increments in 5-HT_{Ext} in hSERT^{WT} (two-way RM-ANOVA; effect of time: $p < 0.001$, $F_{[8,128]} = 4.12$; time $\times$ treatment: $p < 0.001$, $F_{[8,128]} = 3.80$) as well as in hSERT^{ALI/VFL+SI/TT} mice (two-way RM-ANOVA; effect of time: $p < 0.001$, $F_{[8,96]} = 6.60$; time $\times$ treatment: $p < 0.001$, $F_{[8,96]} = 5.38$; Figure 4G–H). The R-citalopram-induced increments in 5-HT_{Ext} were substantially smaller (+50 %) than the effects of escitalopram (+250 %).

Effect of R-citalopram on the effect of escitalopram on 5-HT_{Ext} sensitive behaviors

We further evaluated the potential functional interactions between escitalopram and R-citalopram at the hSERT in the tail suspension and marble burying tests (Borsini et al. 2002; Deacon 2006), two behavioral paradigms generally responsive to acute 5-HTergic drugs (Borsini et al. 2002; Cryan et al. 2005; Deacon 2006; Kobayashi et al. 2008; Njung'e and Handley 1991; Savelieva et al. 2008; Steru et al. 1985). The treatment effects we observed were all modest, plausibly reflecting that the clinically relevant dose used here was lower than those generally reported to produce behavioral effects in these and similar paradigms in mice (Cervo et al. 2005; Cryan et al. 2004; David et al. 2003; Jacobsen et al. 2008; Sanchez et al. 2003a). In the tail suspension test only the combination of 1 mg/kg escitalopram and 2 mg/kg R-citalopram significantly decreased immobility time in the hSERT^{WT} mice (t-test, $p = 0.0369$, versus saline; Figure 5A). In the marble burying test both 1 mg/kg escitalopram alone and in combination with 2 mg/kg R-citalopram significantly decreased marble burying in the hSERT^{WT} mice (t-test, $p = 0.0346$ and 0.0234 , respectively, versus saline; Figure 5B). No significant treatment effects in either assay were observed in the hSERT^{ALI/VFL+SI/TT} mice. Thus, the limited acute behavioral effects we observed pointed to an additive rather than antagonistic action of R-citalopram on escitalopram in mice carrying hSERT, consistent with the microdialysis findings.

DISCUSSION

In this work we addressed an important question in depression treatment biology: why escitalopram is a superior antidepressant to racemic citalopram. Further, our study serves as an exemplar of the possibility of investigating human pharmacology in an animal. Specifically, humanized mice can eliminate potential confounds arising from inter-species

differences in amino acid sequence and molecular interactions between drug and target. Hence, although species differences in downstream and regulatory components remain, wider use of humanized mice in psychopharmacological target research may conceptually provides a higher level of translational fidelity.

We generated mice humanized for the hSERT. In these mice, hSERT replaces mSERT, is expressed at the cell membrane and at the (mouse) eutopic anatomical location, and actively reuptakes 5-HT from the extracellular space. The availability of the hSERT mice enabled us to address the central question of whether R-citalopram antagonizes escitalopram's antidepressant action by directly and immediately antagonizing the hSERT-inhibiting action of escitalopram. Our main read out was 5-HT_{Ext}, the direct mediator of the antidepressant action of SSRIs. 5-HT_{Ext} is a sensitive indicator of SERT activity (Fox et al. 2008; Hjorth et al. 1997; Jennings et al. 2006). 5-HT_{Ext} reflects net 5-HT longitudinal (minutes to hours) release rate well under most conditions since 5-HT appears to be released predominantly extra-synaptically (Bunin and Wightman 1998; DeFelipe and Jones 1988; Descarries et al. 1990). We deliberately focused on *acute* paradigms to avoid potential confounds consequent to the numerous 5-HT system functional adaptations occurring (in for instance SERT and 5-HT_{1A} receptors) in response to *chronic* SSRI treatment (Descarries and Riad 2012; Rossi et al. 2008b). (Previous mechanistic studies on r- and mSERT were likewise acute (Fish et al. 2004; Mansari et al. 2007; Mork et al. 2003; Sanchez et al. 2003b; Sanchez and Kreilgaard 2004; Storustovu et al. 2004).) However, it should kept in mind that SSRI *antidepressant* effects require weeks to emerge, although it is unsettled whether this lag relates to 5-HT_{Ext} dynamics or downstream triggered events (Blier 2003; Harmer et al. 2009).

The key finding in the present studies is that R-citalopram *does not* directly antagonize escitalopram's inhibition of the hSERT^{WT} as determined by microdialysis. It is salient that our humanized mouse data correspond well to two independent human brain imaging studies reporting no inhibition of escitalopram binding to hSERT by R-citalopram following acute, therapeutic doses (Klein et al. 2006; Lundberg et al. 2007). Altogether, these examinations at hSERT suggest that R-citalopram antagonizes escitalopram's antidepressant effect via distinct mechanisms, not related to acute binding interference. This will be expanded on below. Our hSERT microdialysis data contrasts to data obtained in rats (carrying rSERT) reporting that R-citalopram antagonizes escitalopram's 5-HT_{Ext} elevating effect (Mansari et al. 2007; Mork et al. 2003). The reason for this discrepancy is at present not clear. rSERT *vs.* hSERT interspecies difference could obviously be a factor. Substantial species differences in ligand and molecular pharmacology are known from for instance adenosine_{2A}, histamine₃ and neurokinin receptors (Cristalli et al. 2008; Gitter et al. 1991; Ireland-Denny et al. 2001; Iyengar et al. 1997; Leffler et al. 2009). It is possible, though hardly probable, that doses of escitalopram and/or R-citalopram yielding sub- or supraclinical systemic drug levels would have produced a different result. However, the translational relevance of using doses that either produces insufficient pharmacodynamic or, conversely, potential off-target effects or systemic drug levels associated with toxicity is limited. In any case, the premise for the current investigations was the examination of clinically relevant escitalopram and R-citalopram dosings specifically.

A secondary goal was to address the allosteric hypothesis of escitalopram's interaction with hSERT. The ALI/VFL + SI/TT mutations effectively abolish the allosteric action of escitalopram (Neubauer et al. 2006; Zhong et al. 2009) (and Figure 2D). The effect may be indirect since none of the mutated amino acids are predicted to contribute to the putative allosteric site (Plenge et al. 2012). Actually, a very recent report showed that a single mutation, of hSERT amino acid 402 (a residue believed to face the pore vestibule, the supposed locus of the allosteric site) from glycine to histidine, was sufficient to abolish the allosteric action of escitalopram (Plenge et al. 2012). What is important for our studies is that escitalopram allostery is abolished at the hSERT^{ALI/VFL+SI/TT} *ex vivo*, hSERT^{ALI/VFL+SI/TT} expresses at equal levels to hSERT^{WT}, and *in vivo* functionality in the awake resting state appears normal, as evidenced by unchanged baseline 5-HT_{Ext} levels in hSERT^{ALI/VFL+SI/TT} mice. The modestly decreased and increased affinity of escitalopram and R-citalopram, respectively, for hSERT^{ALI/VFL+SI/TT} can potentially impart some limitations on the data interpretation in this mouse model. Nevertheless, we observed a similar 1 mg/kg escitalopram-induced 5-HT_{Ext} response in hSERT^{WT} and hSERT^{ALI/VFL+SI/TT} mice. This strongly indicates that the putative allosteric site is not crucial for escitalopram's binding to the orthosteric site at hSERT *in vivo*, as has otherwise been proposed (Zhong et al. 2011). R-citalopram did not antagonize escitalopram's inhibition of hSERT^{WT}, making the question of the role of the allosteric site in R-citalopram's antagonism of escitalopram moot. R-citalopram actually modestly increased 5-HT_{Ext}, either alone or when added to escitalopram. The parsimonious explanation of these data is that R-citalopram binds hSERT with lower affinity than escitalopram and that the more clear-cut R-citalopram induced enhancement of 5-HT_{Ex} in hSERT^{ALI/VFL+SI/TT} mice reflects the increased affinity for R-citalopram. In fact, it is notable that all the present microdialysis data can be entirely explained by the affinities of escitalopram and R-citalopram for the orthosteric sites on hSERT^{WT} and hSERT^{ALI/VFL+SI/TT}.

We observed relatively small behavioral effects in the tail suspension and marble burying tests in hSERT^{WT} mice. These two tests are generally sensitive to 5-HT_{2C} agents (Crowley et al. 2005; Kobayashi et al. 2008; Njung'e and Handley 1991; Steru et al. 1985). R-citalopram, if anything, had a marginal additive effect to escitalopram, consistent with the microdialysis findings. Larger doses may have produced greater effects (Crowley et al. 2005), but would not be relevant under our precept where use of clinical relevant doses were mandated. No behavioral escitalopram responses were observed in the hSERT^{ALI/VFL+SI/TT} mice, which for the tail suspension test could be explained by generally lower baseline immobility, which in turn could be speculated to relate to an altered hSERT functionality only becoming evident during behaviorally challenged conditions.

It could be hypothesized that R-citalopram antagonizes escitalopram in humans either via mechanisms not involving the hSERT or via mechanisms, allosteric or otherwise, involving hSERT but only becoming apparent after chronic treatment. Functional downregulation of somatic 5-HT_{1A} inhibitory autoreceptors, have long been hypothesized to be a key event in SSRI treatment biology (Artigas et al. 1996). In this light it is interesting that a previous report found that chronic escitalopram, but not the double dose racemic citalopram, downregulates somatic 5-HT_{1A} autoreceptors in the rat (Rossi et al. 2008a). This finding

would be consistent with a notion that R-citalopram attenuates the chronic antidepressant effects of escitalopram. Recent studies suggest that long-term SSRI treatment leads to internalization of SERT (Descarries and Riad 2012; Kittler et al. 2010). Whether SERT internalization is critical for the antidepressant response is not yet clear, but interference with this phenomenon could be a possible locus for R-citalopram modulations of escitalopram pharmacodynamics. Escitalopram is remarkably selective, with very few additional interactions with known molecular targets at clinical concentrations (Sanchez et al. 2003a). However, both escitalopram and R-citalopram possess nanomolar affinity for the sigma₁ receptor with K_i values of 100 and 50 nM, respectively (Sanchez et al. 2003a). Sigma₁ agonists are active in preclinical models of depression, appears to enhance 5-HT neurotransmission and have been in development as antidepressants (Lucas et al. 2008; Maurice and Su 2009), albeit with disappointing outcomes. R-citalopram also has nanomolar affinity for the histamine₁ receptor (Sanchez et al. 2003a), which is an important regulator of sleep. Sleep disturbances are common in depression and may even be causal in the disease (de Bodinat et al. 2010; Thase 2006), although this remains unsettled. Thus, modulation of sigma₁ or histamine₁ receptors could be candidate loci for R-citalopram's antagonism of escitalopram's antidepressant effect; but at this point little data exist to illuminate such hypotheses. Accumulating evidence suggests that phosphorylation regulates SERT on multiple levels, including cell membrane expression, catalytic activity, trafficking and degradation (Ramamoorthy et al. 2011). Indeed, racemic citalopram blocks the ability of 5-HT to limit protein kinase C phosphorylation of SERT *in vitro* (Ramamoorthy and Blakely 1999), and this or similar interactions could be involved in regulation of escitalopram by R-citalopram. Thus, several possible and not mutually exclusive alternative mechanisms underlying the antagonism of escitalopram by R-citalopram can be envisioned.

CONCLUSION

There is strong clinical evidence that R-citalopram inhibits the antidepressant effect of escitalopram (Kennedy et al. 2006; Lepola et al. 2004; Llorca et al. 2005). We here show that R-citalopram does not appear to directly affect the 5-HT_{Ext}-enhancing action of escitalopram at the hSERT, a key finding. Further, the putative allosteric site may not be important for inhibition of hSERT by escitalopram, suggesting the involvement of alternative underlying phenomena. The data presented here in conjunction with the body of previously published data does not as yet provide a comprehensive framework for understanding R-citalopram's antagonistic interactions with escitalopram. Further studies delineating the mechanism(s) by which R-citalopram antagonizes escitalopram could elucidate the mechanism of escitalopram's superior antidepressant action, which in turn could inform about the biology of the antidepressant response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Characterization of hSERT mice. (**A, upper gel**) Amplification of cDNA using primers recognizing both human and mouse SERT cDNA reveals brain expression of SERT mRNA in all genotypes. (**A, lower gel**) Restriction digests of the amplified fragments using MfeI, which cleaves human, but not mouse, SERT cDNA confirm that mSERT is not present in hSERT^{WT} or hSERT^{ALI/VLF+SI/TT} mice. (**B, upper gel**) Amplification of cDNA using primers recognizing both human and mouse SERT cDNA reveals brain expression of SERT mRNA in all genotypes. (**B, lower gel**) Restriction digests of the amplified fragments using

Afl2, which cleaves mouse, but not human, cDNA confirm that mSERT is not present in hSERT^{WT} or hSERT^{ALI/VFL+SI/TT} mice. **(C)** [³H]-escitalopram autoradiography reveals generally similar macroscopic anatomical distribution of SERT protein in mSERT, hSERT^{WT} or hSERT^{ALI/VFL+SI/TT} mice. Binding concentrations used of [³H]-escitalopram corresponding to K_D, as determined in **(D)**, for the three types of SERT were used as indicated. **(D)** [³H]-escitalopram saturation binding (n = 3). **(D, left)** SERT protein levels as deduced from computed B_{Max}. **(D, right)** K_D (n = 3). **(E)** Frontal cortex tissue levels of 5-HT and 5-HIAA (n = 7). *, p < 0.05, vs. mSERT. #, p < 0.05 hSERT^{WT} vs. hSERT^{ALI/VFL+SI/TT}. (One-way ANOVA, Bonferroni's post-hoc test).

Figure 2. Escitalopram dissociation binding. (A) Representative time-concentration plot of retardation of [³H]-escitalopram dissociation from hSERT^{WT} in neuronal membranes by increasing doses of unlabeled escitalopram. (B) Representative time-concentration plot of retardation of [³H]-escitalopram dissociation from hSERT^{ALI/VFL+SI/TT} in neuronal membranes by increasing doses of unlabeled escitalopram. (C) Retardation of [³H]-escitalopram dissociation from hSERT^{WT} in neuronal membranes by increasing doses of unlabeled escitalopram (n = 3). (D) Retardation of [³H]-escitalopram dissociation from

hSERT^{ALI/VFL+SI/TT} in neuronal membranes by increasing doses of unlabeled escitalopram (n = 3). **(E)** Representative time-concentration plot of retardation of [³H]-escitalopram dissociation from hSERT^{WT} in neuronal membranes by increasing doses of unlabeled R-escitalopram added to 3 μ M unlabeled escitalopram. **(F)** Retardation of [³H]-escitalopram dissociation from hSERT^{WT} in neuronal membranes by increasing doses of unlabeled R-escitalopram added to 3 μ M unlabeled escitalopram (n = 3). *, p < 0.05 vs. control. ^, p < 0.05 vs. R-cit 0 μ M (blue). (One-way ANOVA, Dunnett's post-hoc test).

Figure 3.

Escitalopram plasma and brain pharmacokinetics. (A) Time-course of escitalopram plasma levels following acute dosing. Gray-shading delimits the concentration range encountered in the clinic ($n = 3-5$). (B) Maximal plasma escitalopram concentrations (at $T = 30$ min, all cases). (C) Time-course of escitalopram brain levels following acute dosing. (D) Time-course of escitalopram brain:plasma ratios following acute dosing. *, $p < 0.05$, 0.25 mg/kg vs. all other doses. (Two-way ANOVA, Bonferroni's post-hoc test).

Figure 4.

Frontal cortex 5-HT_{Ext} microdialysis. (A) Effect of escitalopram (1 mg/kg) in hSERT^{WT} and hSERT^{ALI/VFL+SI/TT} mice (n = 10). (B) Effect of escitalopram (0.5 mg/kg) in hSERT^{WT} and hSERT^{ALI/VFL+SI/TT} mice (n = 14). (C) Effect of addition of R-citalopram (2 mg/kg) to escitalopram (1mg/kg) in hSERT^{WT} mice (n = 12–16; saline n = 6). (D) Effect of addition of R-citalopram (2 mg/kg) to escitalopram (1mg/kg) in hSERT^{ALI/VFL+SI/TT} mice (n = 8–11; saline n = 7). (E) Effect of R-citalopram (2 mg/kg) alone in hSERT^{WT} mice (n = 4–6). (F) Effect of R-citalopram (2 mg/kg) alone in hSERT^{ALI/VFL+SI/TT} mice (n = 6–7). (G) Effect

of R-citalopram (4 mg/kg) alone in hSERT^{WT} mice (n = 7). **(H)** Effect of R-citalopram (4 mg/kg) alone in hSERT^{ALI/VFL+SI/TT} mice (n = 6–12). #, p < 0.05, hSERT^{WT} vs. hSERT^{ALI/VFL+SI/TT}. *, p < 0.05, R-citalopram vs. no R-citalopram. (Two-way RM-ANOVA, Bonferroni's post-hoc test).

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Figure 5. 5-HT_{Ext} sensitive behaviors. **(A)** Effect of escitalopram (1 mg/kg) alone or in combination with R-citalopram (2 mg/kg) on immobility in the *tail suspension test* in hSERT^{WT} and hSERT^{ALI/VFL+SI/TT} mice (n = 9–12). **(B)** Effect of escitalopram (1 mg/kg) alone or in combination with R-citalopram (2 mg/kg) on *marble burying behavior* (n = 13–16). *, p < 0.05, vs. saline. (T-test).