

DETERMINING THE ROLE OF SEROTONIN IN ANTIDEPRESSANT ACTION, AS
REVEALED UTILIZING THE SERT MET172 MOUSE MODEL

By

Alexander Greer Nackenoff

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Approved:

Eugenia Gurevich, Ph.D.

Ron Emeson, Ph.D.

Ariel Deutch, Ph.D.

Gregg Stanwood, Ph.D.

Randy Blakely, Ph.D.

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TABLE OF CONTENTS

	Page
DEDICATION	ii
ACKNOWLEDGEMENTS	iii
LIST OF TABLES	ix
LIST OF FIGURES	x
Chapter	
I. INTRODUCTION.....	1
Clinical Significance of Depression.....	1
Serotonergic Pathways and Pathophysiology of Depression	2
Early Treatments of Depression and Emergence of Psychopharmacology	8
Biomechanistic Determinations of Antidepressant Efficacy.....	13
The Serotonin Transporter (SERT) and SSRIs	17
The Serotonergic Hypothesis of Depression.....	22
Serotonergic Mechanisms Contributing to SSRI Antidepressant Efficacy.....	24
Serotonergic Evidence of SSRI Efficacy by Direct Receptor Analysis.....	28
SERT-Independent Targets of SSRIs.....	33
The SERT Met172 Mouse Model for Assessing 5-HT Dependence of Drug Action.....	37
Rationale for Thesis Studies.....	41
II. BIOCHEMICAL CHARACTERIZATION OF SERT MET172.....	45
Introduction	45
Materials.....	47
SERT Western Blotting Studies	48
Brain Monoamine Steady State Composition	51
Saturation Uptake Kinetic Analysis of SERT Met172.....	52
Synaptosomal Competition Serotonin Uptake Analysis	55
Conclusions	59
III. ACUTE BEHAVIORAL SENSITIVITY OF SERT MET172 TO SSRI.....	61
Introduction	61
Animal Procedure.....	64
Tail Suspension Test	65
Forced Swim Test.....	66
Conclusions	70

IV. CHRONIC BEHAVIORAL AND BIOCHEMICAL SENSITIVITY OF SERT MET172 TO SSRI	73
Introduction	73
Materials	77
Novelty Induced Hypophagia (NIH)	78
Hippocampal Neurogenesis.....	81
Conclusions	85
V. ANTIDEPRESSANT SENSITIVITY TO THE NOVEL ANTIDEPRESSANT VORTIOXETINE IN SERT MET172.....	89
Introduction	89
Animals	91
Drugs	93
<i>In Vitro</i> Transfected Cell Competition 5-HT Uptake.....	93
<i>Ex Vivo</i> Synaptosomal Competition 5-HT Uptake	96
<i>Ex Vivo</i> SERT Occupancy.....	99
<i>In Vivo</i> Chronoamperometry	102
<i>In Vivo Microdialysis</i>	103
Acute Behavioral Paradigm Animal Procedure	107
Tail Suspension Test (TST).....	108
Forced Swim Test (FST)	110
Novelty Induced Hypophagia (NIH).....	112
Hippocampal Neurogenesis.....	116
Conclusions	120
VI. CONCLUSIONS AND FUTURE EXPERIMENTATION	125
Appendices	135
APPENDIX A. Sensitivity of the Mixed Action SNRI Antidepressant/Pain Medicines Milnacipran and Duloxetine to the SERT Met172 Substitution	136
APPENDIX B. Characterization of the Disrupted Pharmacological Inhibition of Cocaine upon <i>Ex Vivo</i> Derived Synaptosomes from C57Bl/6 Backcrossed SERT Met172 Mice.....	141
APPENDIX C. Adaptation of Novel Fluorescence-Based SERT Uptake Assay for Ninety-Six Well Transfected Cell Format.....	147
REFERENCES	153

LIST OF TABLES

Table	Page
1. Highlighted Major 5-HT Receptors	30
2. Biogenic Amines in SERT Met172	53

LIST OF FIGURES

Figure	Page
1. Location of Serotonergic Raphe Nuclei and Projections.....	3
2. Biosynthetic Pathway of Serotonin.....	5
3. Comparison of the Structures of Isoniazid and Iproniazid	10
4. Structural Comparisons of Iminodibenzyl, Chlorpromazine, and G-22355	14
5. Sequence Alignment of SERT Protein	39
6. Structure of SERT with I172M Substitution.....	40
7. SERT Protein Expression in SERT Met172	50
8. Saturation Uptake Kinetics	56
9. Synaptosomal Competition of 5-HT Uptake	57
10. Tail Suspension Test.....	67
11. Forced Swim Test	69
12. Novelty Induced Hypophagia Test	80
13. Hippocampal Neurogenesis	83
14. Stem cell proliferation in SVZ.....	84
15. Research Strategy for Vortioxetine in SERT Met172	92
16. In Vitro Vortioxetine Competition 5-HT Uptake	95
17. Ex Vivo Vortioxetine 5-HT Uptake Competition.....	98
18. Ex Vivo Vortioxetine SERT Occupancy	101
19. In Vivo Chronoamperometry of Vortioxetine-Inhibited 5-HT Clearance	104
20. In Vivo Microdialysis of Vortioxetine-Induced Elevations in Extrasynaptic 5-HT.....	106

21. Tail Suspension Test with Vortioxetine.....	109
22. Forced Swim Test with Vortioxetine.....	111
23. Novelty Induced Hypophagia with Vortioxetine.....	114
24. Hippocampal Neurogenesis: Proliferation Stimulation by Vortioxetine	118
25. Hippocampal Neurogenesis: Survival Preservation with Vortioxetine	121

CHAPTER I

INTRODUCTION

Clinical Significance of Depression

The earliest depictions of depression-related disorders arose in Hippocrates, *Nature of Man* (Hippocrates), in which he postulated that the presentation of ‘melancholy’ was due to humoral imbalance. Specifically, oversaturation of ‘black bile’ (*melaina kholé*) produced a state currently best described as depression, and whose treatment involved bloodletting in order to restore the balance of the humours. Although these treatments have long since been discredited, these early classifications of depression were foundational for more progressive analyses and diagnosis of depression. Depictions of melancholy began to expand as a defined phenomena of the mind, most notably described at length in 1621 by Robert Burton in *The Anatomy of Melancholy* (Burton). Here, we begin to see parallels to modern diagnostic formulations through more deliberate analysis of the core states of melancholy and depression. Depression is now defined and diagnosed upon the presentation of multiple symptoms including prolonged depressed mood, suicidal ideation, lethargy, anhedonia, and perceptions of low self-worth, as set forth by the Diagnostic and Statistical Manual (DSM-V). Depression symptom presentation can vary across different forms of depression, of which major depression, atypical depression, melancholic depression are most common (Benazzi, 2006).

These historical observations were common not only due to the budding scientific curiosity surrounding depression, but is also due to the high prevalence and persistence of the disorder.

Depression is one of the most common psychological disorders and roughly affects at least 7% of the US population at any given time (Kessler *et al*, 2005), and lifetime prevalence estimates range from ranges of 1 in 10 (Andrade *et al*, 2003) to 1 in 5 (Kessler, 2005). World Health Organization estimates conclude that depression is the primary basis of disability (using the Years Lived with Disability metric) and among the leading contributor to the global burden of disease (2000). Depression is already the second most burdensome disease among 15-44 year olds (measured using the Disability Adjusted Life Years metric), and is projected to further increase in burden across all age groups. The economic impact of depression is dramatic and has been estimated to have reduced economic output in the USA (non-inflation adjusted dollars) by \$43.7 billion in 1990 (Greenberg *et al*, 1993), \$52.9 billion in 2000 (Greenberg *et al*, 2003), and \$210 billion in 2010 (Greenberg *et al*, 2015). Additionally, depression and related mood disorders are the predominant risk factors for suicide attempts (CDC, WISQARS, 2014). In the United States, the suicide rate for adults is 11.3 suicide deaths per 100,000 people with 11 suicide attempts occurring for every suicide death (CDC, WISQARS, 2014).

Serotonergic Pathways and Pathophysiology of Depression

Most serotonergic (5-HT) neurotransmission originates in the raphe nuclei, which are located in the midbrain. Raphe neurons project in both an ascending and descending manner, projecting to the spinal cord, the forebrain, and to major components of the limbic system (**Figure 1**). These serotonergic neurons project to areas implicated in pain processing, fear, emotion, memory formation, and attribution of salience. Serotonin is synthesized from dietary tryptophan, and its delivery into the CNS is the rate limiting step in serotonin synthesis.

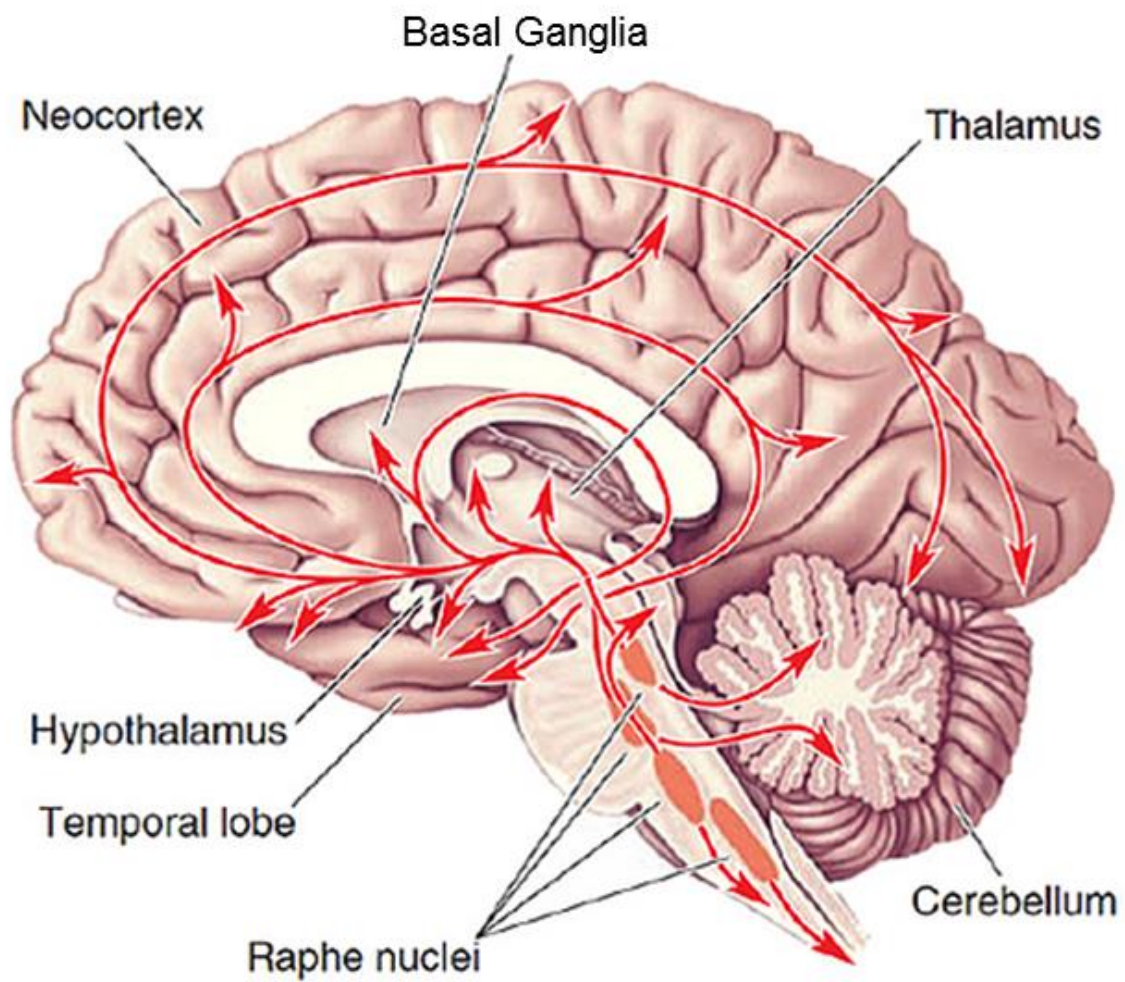


Figure 1. Location of Serotonergic Raphe Nuclei and Projections

Tryptophan is hydroxylated by tryptophan hydroxylase (TPH; TPH2 in the CNS) to form 5-hydroxytryptophan (5-HTP). 5-HTP is then decarboxylated by aromatic amino acid decarboxylase (AADC) to form 5-hydroxytryptamine (5-HT), also known as serotonin. 5-HT is then packaged into synaptic vesicles via vesicular monoamine transporter (VMAT; VMAT2 in CNS), and this 5-HT is released upon neuronal action potential propagation to the nerve bouton, where the synaptic vesicles fuse with the neuronal membrane, releasing the contents into the synapse. This 5-HT is then free to bind and activate pre and post-synaptic 5-HT receptors, which carry the signal propagation of a synaptic signaling event. The 5-HT neurotransmission is primarily terminated by presynaptic serotonin transporters (SERT), which utilize the Na^+/Cl^- ion gradient to transport 5-HT up its concentration barrier in a symporter manner. 5-HT is either then recycled, preserving the energy required for synthesis, or metabolized by monoamine oxidase (MAO) located upon the surface of mitochondria into 5-hydroxyindoleacetic acid (5-HIAA) (**Figure 2**).

SERT (SLC6A4) belongs to the SLC6 gene family of Na^+/Cl^- coupled symporters that include the dopamine transporter (DA; DAT) and norepinephrine transporter (NE; NET), as well as γ -aminobutyric acid transporter (GABA, GAT1), glycine transporter, creatine transporter, and other amino acid transporters (Hahn and Blakely, 2007). SERT rapidly clears 5-HT from the extracellular synaptic cleft following 5-HT synaptic release, resulting in a tightly controlled neural signaling event. The serotonergic system is targeted by multiple drugs of abuse, including 3,4-methylenedioxymethamphetamine (MDMA; 'ecstasy'), lysergic acid diethylamide (LSD), dimethyltryptamine (DMT), psilocybin, as well as cocaine.

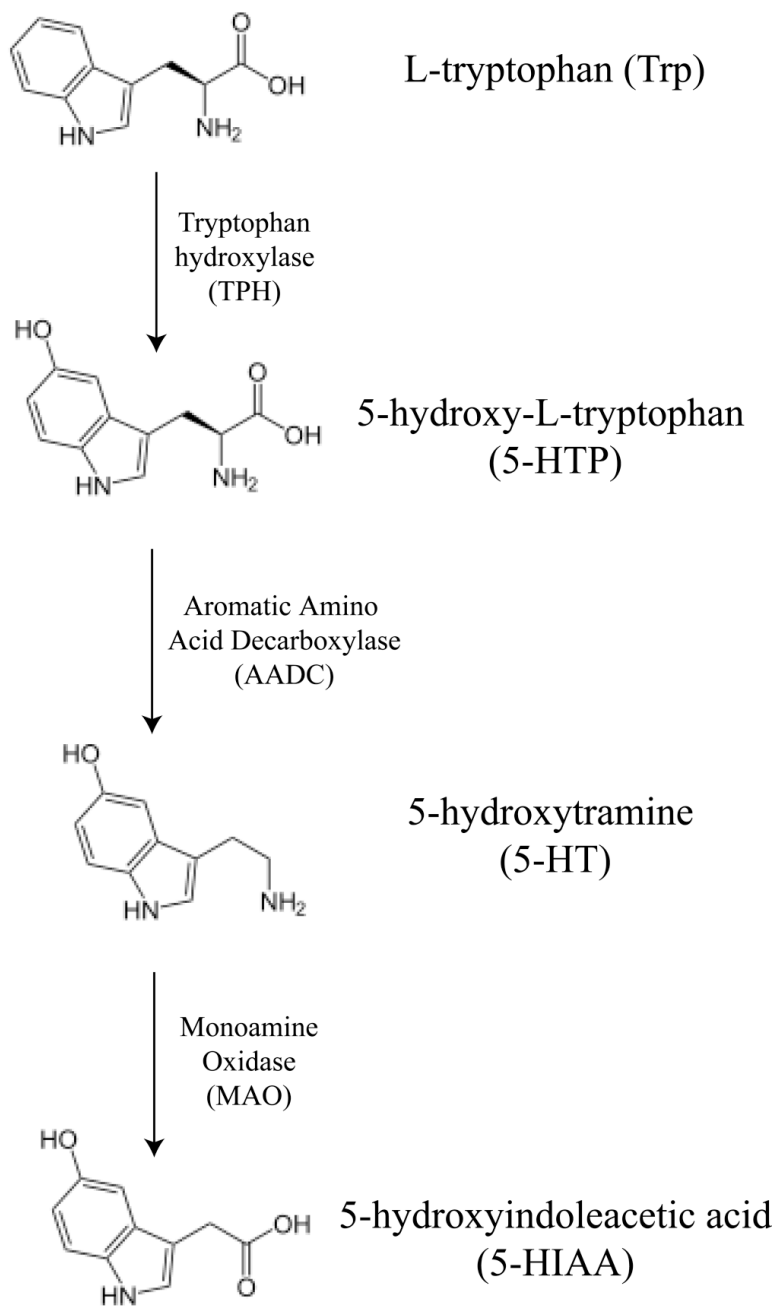


Figure 2. Biosynthetic Pathway of Serotonin. Biosynthetic pathway of serotonin (5-HT), and metabolism by MAO. The rate limiting step in the production of CNS 5-HT is the dietary delivery of tryptophan to the brain. Tryptophan is hydroxylated by TPH, forming 5-HTP, which is then decarboxylated by a general AADC enzyme, forming 5-HT. 5-HT is metabolized primarily by MAO, located intracellularly on the surface of the mitochondrial membrane, into 5-HIAA

While LSD, DMT, and psilocybin act as serotonin receptor agents, MDMA and cocaine target SERT. MDMA acts as a substrate for SERT, and like amphetamine for the dopaminergic system, acts to disrupt vesicular stores of 5-HT, causing 5-HT to pool in the neuronal bouton and allowing non-vesicular release of 5-HT into the synapse via efflux through SERT (Rudnick and Wall, 1992). Cocaine, in addition to major activity at DAT and minor activity at NET, acts as a transporter blocker, much like SSRIs, and with comparable affinity as other SERT blockers (Uhl *et al*, 2002). While most of the research into the behavioral and biochemical actions of cocaine are directed to its major activity at DAT, the activity of cocaine at SERT that could be essential for subtle neuromodulatory activity requisite for certain features of the drug, as for example the actions of cocaine to disrupt regulation of circadian rhythm (Prosser *et al*, 2014) as well as contributions to cocaine use and addiction (Howell and Cunningham, 2015).

Disruptions in the serotonergic system, specifically SERT expression, have been observed in several psychological disorders noted for anxiety phenotypes, including obsessive compulsive disorder, autism, and depression. Polymorphisms in the SERT promoter region have been discovered in human populations, and it has been shown that the short allele of the SERT promoter produces reduced transcription efficiency of SERT (Lesch *et al*, 1996). People who are homozygous or heterozygous carriers of the short allele of SERT display more depressive symptoms compared to those who were homozygous for the long allele promoter (Caspi *et al*, 2003). It was also shown in the same study that those with two copies of the short allele displayed increased sensitivity to life stressors, measured by risk for the development of depression, thus showing a gene-by-environment interaction for depression risk factors (Caspi *et al*, 2003). As noted previously, several findings indicated that serotonergic tone was dramatically reduced in depressed individuals. Pharmacological intervention strategies have shown that drugs that

increase serotonergic tone are effective antidepressants. First generation tricyclic antidepressants target both SERT and NET, but had some untoward cardiovascular side effects due the enhancement of the noradrenergic (NE) system. Thus new versions of antidepressants were generated and designed to remove the NE component, and thus spawned the second generation of antidepressants, the serotonin selective reuptake inhibitors (SSRIs).

The primary treatment for those afflicted with depression diagnoses are serotonin (5-HT) selective reuptake inhibitors (SSRIs) (Blakely *et al*, 1998). SSRIs act to block the 5-HT transporter (SERT; 5-HTT), and by doing so prolongs 5-HT signal duration by inhibiting synaptic 5-HT clearance, and it is thought that this 5-HT signal enhancement, over time, produces antidepressant effects in the clinic. Patients diagnosed with depression, however, do not uniformly respond to SSRIs. In fact, roughly only two thirds of patients respond to the first administered SSRI antidepressant (Ananth, 1998; Souery *et al*, 2006), roughly 20% of afflicted patients are completely resistant to all SSRI antidepressant pharmacotherapies (Ananth, 1998; Souery *et al*, 2006), and full remission of depression symptoms is not achieved in 50-75% of treated patients (Souery *et al*, 2006). Moreover, even in those patients who do respond to SSRI pharmacotherapy, no clinically significant effects occur until after 4-8 weeks of administration (Ananth, 1998; Warden *et al*, 2007). These clinical results raise questions regarding the serotonergic basis of depression and the mechanism of SSRI antidepressant efficacy, especially considering the mechanisms by which we know SSRIs function acutely are obviously not immediately responsible for clinical efficacy, as central nervous system (CNS) SERT inhibition by SSRIs occur in minutes whereby clinical efficacy requires weeks of SSRI treatment. Simply, this temporal disconnection of pharmacological effects and clinical efficacy illuminates that our understanding of the mechanisms of antidepressant suggests that our appreciation for how SSRIs produce

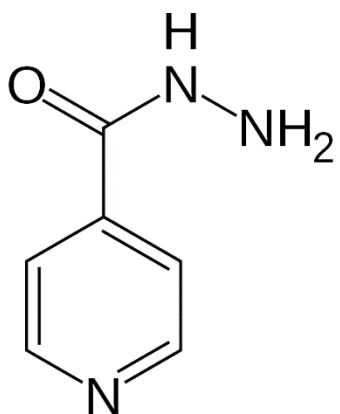
antidepressant actions are possibly more complicated than simply SERT antagonism. Alternatively, it could also mean that the adaptive changes following repeated SSRI administration require lengthy periods of time to produce an antidepressive state, all while being dependent upon 5-HT signal enhancement. In order to better understand the complex nature of SSRI antidepressant efficacy and the 5-HT hypothesis of depression, let us explore how this theory came to be and how SSRIs were created to treat major depressive disorder.

Early Treatments of Depression and Emergence of Psychopharmacology

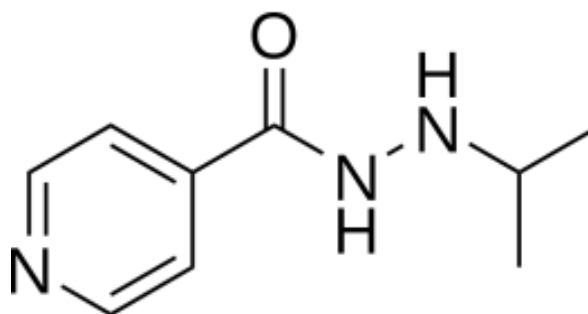
The treatments that we develop for disorders of the body are largely crafted based upon the presentation of the disorder and the nature in which it debilitates the individual. While we can bandage bleeding wounds and brace broken legs, how does one remedy disorders of the mind? The presentation of depression and other mood disorders are much more gradient than physical maladies. There is no normal amount of regular human bleeding; a leg is either broken or it is not. Diagnosis of such mood disorders is complicated enough given that it requires an assessment of abnormal presentation of semi-regular human conditions. In short, the treatments for disorders of the mind cannot be as simple as disorders of the physical form. Unsurprisingly, the early treatment options for depression were highly experimental and questionably effective. While the most effective treatments stemmed from counseling efforts to find the psychological ‘root causes’ of the disorder (Freud, 1953), some aimed to reset the mind through convulsions (Payne and Prudic, 2009) to a normal state as with electroshock therapy (while successful, its mechanism of action is still largely unknown), and others randomly tried a swath of biologically active chemical agents including lithium salts (Cade, 1949), barbiturates, amphetamines, and opiates, among other

questionably effective agents including lactic acid, succinic dinitrite, and malonic nitrite (López-Muñoz and Alamo, 2009).

Happenstance discoveries were not uncommon in this era of experimentation, though the careful observation and analysis of behavioral outcomes is responsible for the extraction of utility and development of future treatments. The field of antidepressant pharmacology stems from many sequential chance discoveries. The origins of antidepressant pharmacology can be traced to a number of instances, though the importance of early chemistry must not be understated. German chemists Hans Meyer and Josef Malley synthesized isonicotinyldiazide in 1912, mostly for the purpose of academic chemical derivatizations of hydrazine hydrate (López-Muñoz and Alamo, 2009). Isonicotinyldiazide was largely forgotten until the 1950s when German World War II stockpiles of hydrazine (utilized as rocket fuel (Sandler, 1990)) were distributed to scientists and industrial research groups at low cost for academic pursuits, when isonicotinyldiazide was resynthesized and discovered later to have anti-tubercular properties in trials in 1951 by Harry Yale at Squibb and Herbert Fox at Hoffmann-La Roche (Sneider, 1985), and was later given the name Isoniazid (López-Muñoz and Alamo, 2009). Isoniazid spawned chemical derivatives for optimal human efficacy against tuberculosis, one of them being iproniazid (**Figure 3**)**Error! Reference source not found.** It was this drug that was given to a small tuberculosis patient group to test this hypothesis of increased efficacy over isoniazid in New York in 1952. In this study, the practitioners observed much more pronounced psychological cognitive effects that they attributed to a side effect of the drug (Selikoff *et al*, 1952). Because of these reports on psychostimulation, iproniazid was later directly studied for its mood elevating properties as a direct endpoint of a clinical study, and was shown to act as an antidepressant, though this terminology was not utilized until later (Ayd, 1957; Crane, 1957).



Isoniazid



Iproniazid

Figure 3. *Comparison of the Structures of Isoniazid and Iproniazid.* Comparison of the structures of isoniazid and its derivative iproniazid. Isoniazid originated as a treatment for tuberculosis. Iproniazid was developed as an alternative treatment molecule, but also produced antidepressant-like effects among tuberculosis patients, an effect that was not observed with isoniazid. It was discovered later that iproniazid acted additionally as a monoamine oxidase inhibitor (MAO; MAOI). MAO acts to degrade and metabolize 5-HT, and MAOIs prevent this 5-HT metabolism. The net effect of MAOIs with respect to 5-HT signaling is to prolong the serotonergic tone indirectly by increasing 5-HT levels. This mechanism is proposed for the antidepressant effect of MAOIs, and helped the 5-HT hypothesis of antidepressant effects in addition to the 5-HT hypothesis of depression.

Iproniazid was then subjected to clinical studies using patients with direct indications of depression and mood disorders, and following their successful completion, the drug was no longer solely prescribed for tuberculosis and was administered to an upwards of 500,000 people (Sneider, 1985), indicating the large unmet pharmacological need for antidepressant medications. It was discovered that iproniazid coincidentally, but not isoniazid, could inhibit the recently discovered monoamine-oxidase (MAO) enzyme (Zeller *et al*, 1952), whose activity includes the oxidation and metabolism of 5-hydroxytryptamine (5-HT; serotonin) into 5-hydroxyindoleacetic acid (5-HIAA). By inhibiting MAO, iproniazid could slow the metabolism of biogenic amines, including 5-HT into 5-HIAA and trigger increases in brain levels of 5-HT, though it was unclear at the time whether this mechanism was responsible for the mood elevating qualities of iproniazid. Concurrently, it was discovered that the antihypertensive drug reserpine could produce depressive-like symptoms as a side effect, and was also found to reduce 5-HT tissue levels (Pletscher *et al*, 1955). Given these findings, effort proceeded in the development of intentionally designed MAO inhibitors (MAOIs) as antidepressant drugs (Ban, 2001; Sandler, 1990). These MAOIs found great clinical and commercial success, and further developments for lower toxicity (eg. reversible inhibitors) and toward pharmacological specificity for MAO-A (responsible for the metabolism of serotonin and norepinephrine) vs. MAO-B (largely responsible for metabolism of dopamine and trace amines) leading to improved versions of MAOIs for the treatment of depression. MAOIs, despite pharmacological advancements, were limited in effective dose range by drug interactions and toxicity, and additionally proved not to be the chemical magic bullet for the treatment of depression that clinicians had hoped; there were significant populations of people who met clinical thresholds for depression diagnoses, but were not successfully treated with MAOIs. Though the development of these first generation antidepressants represented a momentous advancement in

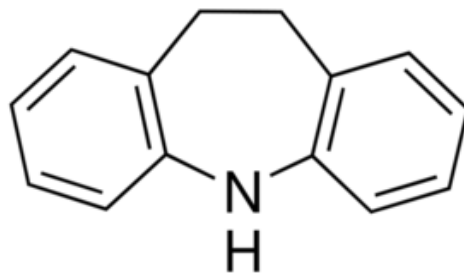
the field of psychopharmacology, specifically advancing the idea that modulation of brain chemistry could alter and alleviate psychological maladies, previously thought to be only remedied through intensive psychotherapy. The development of MAOI antidepressants indicated that it was possible to treat these disorders through chemical means, though their limited efficacy indicated incomplete knowledge of the disorders, but represented the most efficient means of pharmacological treatment to date. The early determination of the pharmacological actions impacting 5-HT and NE homeostasis would prove useful for future drug development and hypotheses related to the origins and optimal treatment of depression.

Another important trajectory in the development of antidepressant pharmacology stems from the early investigations of antihistamines and antipsychotics. Based upon some research implying that antihistaminergic drugs could be useful as antipsychotics, researchers at Geigy Pharmaceuticals began synthesizing derivatives of iminodibenzyl (a tricyclic ring structure originally synthesized for potential use as a dye in textiles) (Schindler and Häfliger, 1954). These researchers found that functional consequences of their derivatives were largely due to the composition of the lateral chain additions of the molecule. One of these derivatives (G-22150) was sent to a local clinician, Ronald Kuhn, for testing in schizophrenic patients, but it was discovered to have poor efficacy. Interestingly, Kuhn noted an unexpected elevation of mood in the treated individuals (Shorter, 2008), though Geigy decided not to pursue the project. Interest was renewed when positive results were announced in 1952 for the efficacy of another molecule, chlorpromazine (later to be named thiorazine), in the treatment of schizophrenia. In the wake of these findings, Geigy sent Ronald Kuhn more chemicals to test in his schizophrenic patient population. In 1956 they sent him G-22355, which possesses the same tricyclic ring structure as G-22150, but contains the same lateral N,N-dimethylpropanamine chain as chlorpromazine. G-

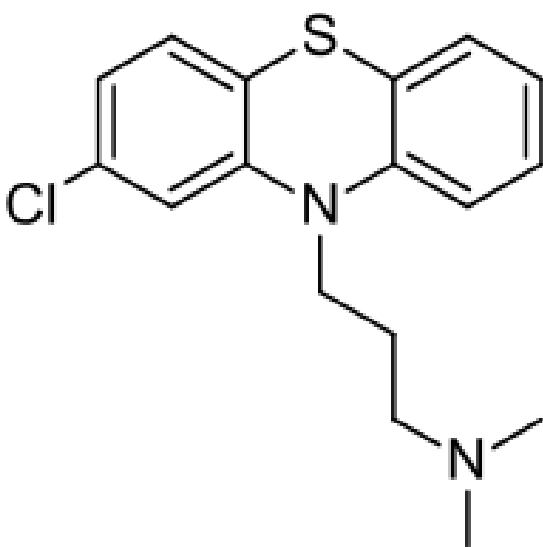
22355 unfortunately lacked any appreciable efficacy in schizophrenic patients, though Kuhn noted remarkable mood elevating effects in a smaller group of depressed patients (Kuhn, 1957). A larger study was performed to confirm Kuhn's suspicions, and the positive results were presented in 1957 (Kuhn, 1958). This compound was renamed to imipramine, and represented the first tricyclic antidepressant (**Figure 4**). Much like the discovery of iproniazid and the MAOIs, these results were entirely serendipitous.

Biomechanistic Determinations of Antidepressant Efficacy

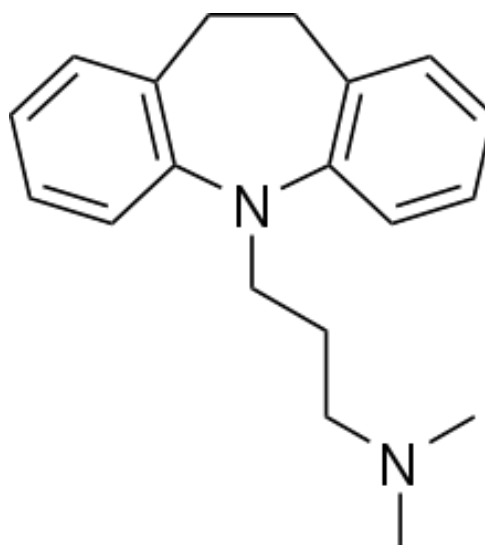
After the introduction of imipramine and its clinical efficacy were widely observed, many other tricyclic derivatives soon followed onto the market to ride the coattails of the advancements made by imipramine. The clinical viability of iproniazid and imipramine further developed and solidified this novel concept that chemical agents could impact brain function and psychiatric disorders, using this new concept of biological psychiatry. This hypothesis triggered curiosities into the biochemical mechanisms of actions for these chemicals and how they produced their neuropsychiatric effects. Through the development of new imaging-mediated quantification techniques via the creation of spectrophotofluorimetry, it was then possible to accurately detect levels of monoamines in collected samples, which were suspected to be involved in the actions of these antidepressants. Through the use of this technique, it was observed that the previously identified phenomenon of reserpine causing depressive-like behaviors in animals correlated with the depletion in 5-HT and NE (Brodie *et al*, 1955; Curzon, 1990). Further studies determined that pretreatment of iproniazid alone could block the reserpine-induced reductions in brain serotonin as well as the sedative effects of reserpine (Chessin *et al*, 1957).



Iminodibenzyl



**Chlorpromazine
(Thorazine)**



**G-22355
(Imipramine)**

Figure 4. Structural Comparisons of iminodibenzyl, chlorpromazine, and G-22355. Comparisons of the structures of iminodibenzyl, chlorpromazine (better known as the antipsychotic thorazine), and G-22355 (better known by its later name imipramine). Iminodibenzyl was derivitized, seeking new antipsychotic, hypnotic, or antihistaminergic psychotropic drugs. One of these derivatives was crafted intelligently, based upon the southern nitrile tail of chlorpromazine, already known to be an effective clinical antipsychotic. The resulting compound G-22355 did not possess any antipsychotic effects, though patients treated with the compound displayed antidepressant effects after repeated administration. These effects were confirmed later with antidepressant evaluations as proper endpoint analyses, and stimulated the development of tricyclic antidepressants (TCAs). It was later discovered that TCAs block the reuptake of serotonin and norepinephrine.

Additionally, iproniazid was shown to be able to increase levels of brain serotonin and norepinephrine (Besendorf and Pletscher, 1956). These studies were the first to suggest that certain neuropsychiatric disorders could be due to imbalance in brain monoamines, either due to low levels of serotonin, or norepinephrine, or both.

It was at this point that certain advances in the field were made surrounding imipramine and the now growing family of other tricyclic antidepressants (TCAs). In a series of studies utilizing new radiochemical labelling, Julius Axelrod showed that pretreatment of imipramine and other TCA derivatives could block the uptake of [³H]-norepinephrine into nerve endings in peripheral tissues (Axelrod *et al*, 1961) as well as in intact rat brain (Glowinski and Axelrod, 1964). These studies supported the hypothesis that norepinephrine was being released and reabsorbed in a similar manner that had been observed in the periphery, and additionally, that imipramine and these TCAs functioned as reabsorption blockers. Earlier studies had shown that imipramine could reverse the pro-depressive effects of reserpine, and that these effects were lost when catecholamines (which include dopamine and norepinephrine) were depleted using alpha-methylparatyrosine (AMPT; a selective antagonist of tyrosine hydroxylase, which is the rate limiting enzyme for the biosynthesis of the catecholamines dopamine and norepinephrine), indicating that norepinephrine was essential for the behavioral actions of imipramine. Taken together, these studies were seminal for the development of the norepinephrine hypothesis of depression, pioneered by Joseph Schildkraut, given that promoting norepinephrine signaling could produce antidepressant-like effects (Schildkraut, 1965). Clinical depression was posited to be the result of norepinephrine deficits, either due to levels of the hormone or another requisite component on the signaling cascade (Schildkraut, 1965). This hypothesis inspired such terms as ‘chemical imbalance’ as a means to describe depression that is still somewhat in use today.

In parallel with the norepinephrine-directed line of research, converse efforts were being taken to test the other known functions of imipramine and the TCAs, namely their ability to modulate the serotonergic system. In parallel work corroborating the potential role of serotonin depletion in the pro-depressive behavioral actions of reserpine, studies with MAOIs showed that the pre-administration of tryptophan, a biosynthetic precursor to serotonin, could amplify the antidepressant-like effects of MAOIs in animals (Coppen *et al*, 1963). Mimicking the work performed upon norepinephrine uptake blockade, Arvid Carlsson showed that imipramine could also inhibit the uptake of serotonin into nerve terminals (Carlsson *et al*, 1968). Importantly, Carlsson noted in this study that the celebrated effect of imipramine to block norepinephrine uptake was much less potent than imipramine's ability to inhibit the uptake of serotonin, suggesting that imipramine's actions on serotonin homeostasis might be more significant clinically than its actions pertaining to norepinephrine. While these studies were highly important to the field from technical standpoints, more work was needed to link how imipramine and the TCAs were able to bring about their antidepressant effects, and by which neurotransmitter to perform those actions.

The serotonin hypothesis of depression (and conversely the proposed mechanism for imipramine and TCA efficacy) began taking shape from these findings. They were also supported by various observations linking reduced concentrations of serotonin and its metabolite 5-HIAA in post-mortem brain samples of completed suicide victims (Shaw *et al*, 1967), as well as demonstrations of reduced cerebrospinal fluid levels of 5-HIAA in living depressed patients (Ashcroft *et al*, 1966). Moreover, there were mild clinical antidepressant effects of serotonin precursors tryptophan and 5-hydroxytryptophan (Coppen, 1967). Given these findings, a research team was assembled by Eli Lilly to embark on the development of new antidepressants targeting

the inhibition of serotonin reuptake pumps. These serotonin reuptake pumps were only known to exist through their function by this point and were not yet available in isolated nor concentrated preparations. A crucial development on this front was the invention of a technique by Victor Whittaker for the purpose of isolating and studying nerve terminals for the study of synapse-enriched cellular components (Whittaker *et al*, 1964). Whittaker developed a technique by which these neurons would be mechanically ruptured at the point of disrupted cell membranes, and these budded nerve terminals would preferentially close back upon themselves encapsulating a functional nerve terminal. Through buffer optimization and centrifugal separation, Whittaker could isolate these nerve terminals, termed ‘synaptosomes’ (Whittaker *et al*, 1964).

At this point, it was known that these serotonin reuptake pumps likely resided and were enriched in nerve terminals. Solomon Snyder adapted the synaptosomal technique to concentrate monoamine containing synaptosomes from the rest of the homogenized brain material for the purpose of studying synaptic reuptake pumps (Gfeller *et al*, 1971; Kuhar *et al*, 1970). Using these synaptosomes, Snyder and colleagues were able to determine with high resolution the kinetics of specific serotonin uptake through these reuptake pumps for the first time (Wong *et al*, 1973). Using the synaptosome preparation, Lilly’s team embarked on a directed high-throughput path towards rationally designing a serotonin reuptake blocker, leading to the development of the first serotonin selective reuptake inhibitor (SSRI), later to be known as fluoxetine (Prozac) (Wong *et al*, 1974).

The Serotonin Transporter (SERT) and SSRIs

It is important to note that these discoveries and creation of the first specific serotonin reuptake inhibitor occurred only using nerve terminal concentrated populations and without the

aid purified transporter proteins or cloned transporter cDNAs. Through analyses of serotonin uptake kinetics (Wong *et al*, 1973) and pharmacological inhibition of serotonin uptake (Carlsson *et al*, 1968; Wong *et al*, 1974), it was likely that there was only a single specific serotonin transport pump responsible for the uptake of serotonin, though there was evidence for separate low affinity uptake mechanisms apart from this serotonin transporter (Shaskan and Snyder, 1970).

Researchers began to investigate the regional composition of this purported serotonin transporter and the serotonin pathway, by which SSRIs produce their behavioral effects. Initial early investigative efforts were built upon through technological advancements whereby studies could utilize autoradiographic and electron microscopic imaging to investigate where intraventricularly injected [³H]5-HT could be actively absorbed in the intact rodent brain (Aghajanian and Bloom, 1967), notably indicating the localization of the serotonergic neurons (here also showing that different types of neurons absorb serotonin or norepinephrine, respectively), and that serotonin [and norepinephrine] are predominantly absorbed into nerve terminals. Though it was known in some capacity that certain brain regions were more enriched with serotonin, this study confirmed those finding by finding high rates of [exogenous] serotonin absorption in the midbrain (Aghajanian and Bloom, 1967). Another study found high rates of serotonin absorption in rat brain slices containing hypothalamus, striatum, and cerebral cortex, as well as demonstrating a sodium dependent uptake process for both norepinephrine and serotonin uptake (Shaskan and Snyder, 1970). Other studies that utilized [³H]imipramine binding to localize fluoxetine competition sensitive serotonin transporter sites corroborated these findings, especially when known serotonin stores were localized to nerve terminals, by indicating high imipramine binding at midbrain, cortex, striatum, hypothalamus, and hippocampus, among others, as well as

corroborating low presence of serotonin in the cerebellum (Fuxe *et al*, 1983; Palkovits *et al*, 1981; Raisman *et al*, 1980).

Initial attempts to visualize the location of serotonin in the brain were aided by the creation of the histofluorescence technique, pioneered by Falck and Hillarp allowed for the fluorescent detection of endogenous monoamines in brain tissue (Carlsson *et al*, 1961; Falck *et al*, 1982; Falck and Torp, 1961). These approaches were adapted for the detection of serotonin, though this technique was plagued with limited fluorescent detection time (Sladek *et al*, 1974; Smialowska, 1975; Stefano and Aiello, 1975). Another approach was taken to identify the location of serotonergic neurons via creation of antibodies against serotonin (Steinbusch *et al*, 1978). Immunohistochemistry approaches improved over earlier attempts to localize serotonergic neurons by targeting biosynthetic enzymes that synthesize serotonin (eg. tryptophan hydroxylase). In this effort, Steinbusch and colleagues created an antibody to serotonin using bovine serum albumin (BSA) linked to serotonin, injected into rabbits for polyclonal antibody creation, isolated the antibodies against serotonin (via affinity chromatography and saturation of antibodies against BSA with BSA), and applied that diluted antibody (recognizing serotonin) to rodent brain sections. Binding of the antibody to formaldehyde immobilized serotonin was detected with fluorescently labeled sheep anti-rabbit antibody [for photodetection methods], and revealed serotonin specific labeling and positive affirmation that the location of serotonin containing neurons and projections, including axons in the caudate nucleus (of the striatum), the dorsal horn of the spinal cord, and the raphe magnus nucleus (Steinbusch *et al*, 1978). Using this technique, Steinbusch further examined in fine detail the regional distribution of serotonin containing neurons and projections throughout the rodent brain (Steinbusch, 1981).

While these studies were helpful to determine the likely site of action of SSRIs and the neurological subset of cells that they seemed to be acting upon, they did not solidly conclude the existence of the purported serotonin transporter, the site that was proposed that these TCA and SSRI antidepressants were acting. The discovery and identification of this protein gene product would further aid drug development for future antidepressants as well as highlight a new target for the study of the manifestations of depression and mood disorders. This opportunity was greatly advanced by the cloning of cDNAs encoding the norepinephrine transporter (NET) (Pacholczyk *et al*, 1991) and the γ -aminobutyric acid transporter (GABA; GAT1) (Guastella *et al*, 1990). The effort to clone the serotonin transporter was initiated using polymerase chain reaction (PCR) primers targeted to conserved regions of NET and GAT1, yielding a range of PCR products, among them partial clones of unknown genes that were sequenced and found to have significant conservation of amino acid sequence conservation to NET (Blakely *et al*, 1991). One of these cDNA clones, rMB6-25, was produced in radiolabeled form to visualize the distribution of the complementary mRNA via *in situ* hybridization in rodent brain slices. Intense labeling was identified in the dorsal raphe, which had been known to contain highly enriched in serotonin and serotonin containing cell bodies (Steinbusch *et al*, 1978). A separate sequence of rMB6-25 was used to screen a rat cDNA library (from brainstem) using plaque hybridization. A positively identified plaque (BS4E-10) was isolated, and the phage insert cloned into a vector for mammalian cell expression. This vector was then transfected into HeLa cells, and these cells displayed saturable, Na⁺ dependent 5-HT uptake, as well as sensitivity to all major reuptake inhibitors, with known SSRIs (Blakely *et al*, 1991). The rMB6-25 identified cDNA was designated as the rat serotonin transporter (rSERT).

When the primary sequences of the cloned transporters were compared, rSERT, hNET, and rGAT1 were found to share highly conserved regions, particularly those suggested to encode transmembrane alpha-helices. Additionally, some commonalities displayed among rSERT and hNET shared sequences are not present in rGAT1, which could be due to relative similarities of their substrate, as well as their shared interactions with TCAs (Blakely *et al*, 1991). The proposed structure of rSERT contains 12 transmembrane domains, cytoplasmic N- and C-termini (with a predicted phosphorylation site on the N-terminal tail, distal from the membrane), and a large extracellular loop 2 (with multiple predicted glycosylation sites). Other studies validated this model were purely using biochemical methods to validate SERT topography, until a three dimensional crystal structure was elucidated for the bacterial leucine transporter (LeuT), a genetic member of the solute carrier family (SLC), of which SERT and NET are members (Yamashita *et al*, 2005). The LeuT structure provided critical clues for the tertiary structure for these SLC transporters, with respect to transmembrane domain arrangement as well as the critical orientation and amino acid residues necessary for substrate recognition. Given familial level sequence homology between LeuT and hSERT, the three dimensional crystal structure of LeuT was used to design a predicted three dimensional model for SERT. Specifically, the electron space map the comprises the LeuT structure was used as a shell by which the linear sequence of SERT was inserted into the model, and given the known structure of LeuT and the presence of its domains in the structure, a homology based three dimensional structure for SERT was established (Jørgensen *et al*, 2007; Kaufmann *et al*, 2009; Ravna *et al*, 2006; Tavoulari *et al*, 2009). Based upon this predictive mapping and *in vitro* data confirmation, it was determined that Ile172 and Tyr95 were integral for the high affinity binding of SSRIs at SERT, among other structural discoveries (Henry *et al*, 2006; Kaufmann *et al*, 2009; Ravna *et al*, 2006; Tavoulari *et al*, 2009).

The Serotonin Hypothesis of Depression

The link between 5-HT and depression had been initially postulated based upon the antidepressant efficacy of enhancing serotonergic signaling via serotonin reuptake inhibitors (TCAs and SSRIs). As such, researchers began to investigate the converse argument, namely whether clinical depression correlated with the presentation of lowered basal serotonin, ie. whether depression was due to a chemical imbalance and deficits in serotonin. These clues led investigators to examine whether depressed individuals display any aberrations in 5-HT homeostasis.

The first observations were directed towards patients with the most severe forms of depression. Researchers examined the brain concentrations of 5-HT in suicide victims and control samples; multiple research groups found significant reductions in brain 5-HT in the suicide victims compared to the control samples (Lloyd *et al*, 1974; Pare *et al*, 1969; Shaw *et al*, 1967). Further investigations found that cerebrospinal fluid (CSF) levels of 5-hydroxyindoleacetic acid (5-HIAA; the primary metabolite of 5-HT) were inversely correlated with the severity of depression in a living sample, and overall levels of CSF 5-HIAA in depressed individuals were lower than a control sample (Asberg *et al*, 1976). Moreover, among those in the study that had attempted suicide, CSF 5-HIAA levels were inversely correlated with the severity of the violent nature of the suicide attempt (Asberg *et al*, 1976). Further analyses discovered links to depression and the expression of the serotonin transporter (SERT), which is a key component in the termination of 5-HT signaling in the brain.

SERT is also present in the gut and platelets, and is involved in non-psychological processes of gut motility and platelet aggregation, respectively (Mawe and Hoffman, 2013). These other sites of SERT expression can be useful for relatively non-invasive biomarker screens directed

at SERT related disorders. This approach was taken to examine SERT expression levels in untreated depressed individuals (Briley *et al*, 1980). Blood was collected from the untreated depressed sample along with untreated non-depressed age matched individuals, and SERT expression was measured using [³H]-imipramine binding. It was found that the depressed sample had significantly lower maximal [³H]-imipramine binding than the control sample, meaning that SERT expression on platelets was lower in the depressed sample than the non-depressed control sample (Briley *et al*, 1980). Several studies have replicated this finding, and it was presumed that similar mechanisms could be involved that control SERT expression in the brain as are in platelets, implicating the potential for using platelet SERT expression as a biomarker approach for the diagnosis of depression (Owens and Nemeroff, 1994). Other approaches seemed to support the assertion that platelet SERT expression was not only decreased in depressed individuals, but could be predictive of brain SERT expression. Post mortem analyses were able to confirm these suspicions, and showed that SERT expression was significantly reduced in depressed individuals compared to a control sample, using [³H]-cyanoimipramine autoradiographic imaging techniques (Arango *et al*, 1995; Mann *et al*, 2000; Owens and Nemeroff, 1994). More advanced techniques utilizing positron emission tomography in addition to radiolabeled SERT ligands found similar reductions in SERT expression in living untreated depressed individuals compared to untreated non-depressed control samples (Malison *et al*, 1998; Willeit *et al*, 2000). Additionally, it was found that dietary restriction of tryptophan (a 5-HT precursor) could trigger a relapse of depression in an at-risk patient sample (Bremner *et al*, 1997; Delgado *et al*, 1990; Smith *et al*, 1997). Patients that had been successfully treated with SSRIs were given diets that were devoid of tryptophan, an obligate necessary dietary amino acid that is synthesized into serotonin. These patients rapidly relapsed into a depressive-like state. These findings sculpted the hypothesis that depression either

coincided with—or is caused by—disruptions in serotonergic tone. Pharmacological approaches would then aim to restore serotonergic tone for the treatment of depression; the most direct mechanism for accomplishing this seemed to be for targeted inhibition of SERT.

Serotonergic Mechanisms Contributing to SSRI Antidepressant Efficacy

Much of the initial work on the evaluation of antidepressant drug action began with the examination of serotonergic neuronal morphology and the macronetwork of serotonergic fiber innervations. Following the discovery of SERT and the regional distribution of serotonergic neurons and fiber projections, attention was turned to the identification of important biological responses and requirements involved in the antidepressant response. Serotonergic raphe neurons mediate their own firing rate via autoreceptors, due in part to the somatodendritic serotonin 1A (5-HT_{1A}) autoreceptor (Hjorth *et al*, 2000). 5-HT_{1A} receptors are 7-transmembrane G-protein coupled receptors (GPCRs) that couple to G $\alpha_{i/o}$, which are inhibitory in nature, by preventing neuronal firing and 5-HT vesicle mediated release (Barnes and Sharp, 1999). These autoreceptors counteract the actions of SSRIs by inhibiting raphe firing rates after activation by 5-HT; when SSRIs mediate the elevation of extracellular levels of 5-HT, this in turn mediates the increased activation of these inhibitory autoreceptors, thus offsetting the actions of SSRIs (Blier, 2003). Much attention has been given to this issue, as well as the adaptations in these systems that occur alongside behavioral/clinical efficacy. It has been observed that 5-HT_{1A} receptors are eventually desensitized over the course of a few weeks, which could explain the delayed efficacy of SSRIs (Blier, 2003; Blier *et al*, 1987; Hensler, 2002; Le Poul *et al*, 1995). Interestingly, 5-HT_{1A} receptor desensitization was not the result of decreased surface expression of 5-HT_{1A} receptor, but rather

due to a decreased coupling efficiency, measured by 5-HT_{1A} receptor-stimulated [³⁵S]GTPγS binding (Hensler, 2002). The hypothesis that 5-HT_{1A} receptor desensitization is required for the efficacy of SSRIs was further tested after the creation of the 5-HT_{1A} receptor knockout mouse. These mice displayed reduced basal immobility in the tail-suspension test (TST) compared to WT controls, and displayed no further decrease in immobility after fluoxetine or paroxetine treatment at doses that produced effects in WT mice (Mayorga *et al*, 2001). These data show that mice lacking 5-HT_{1A} receptors may display a basal ‘antidepressive’ phenotype, but also suggest that 5-HT_{1A} receptors may be necessary for the acute effects of SSRIs (Mayorga *et al*, 2001). 5-HT_{1A} receptor KO mice are also unresponsive to chronic SSRI administration in the novelty-suppressed feeding paradigm (Santarelli *et al*, 2003). The 5-HT_{1A} receptor KO mouse globally eliminates 5-HT_{1A} receptors, which includes not only autoreceptors but postsynaptic receptors as well, so the behavioral effects of 5-HT_{1A} receptor KO mice cannot be solely attributed to the lack of raphe neuronal autoinhibition. These data illustrate one potential explanation of delayed SSRI efficacy, and overall, indicate the importance of 5-HT_{1A} receptors on the behavioral efficacy of SSRIs.

Another important observation provides further clues to the nature of neurological aberrations that are a result of—or caused by—depression. It was reported that hippocampal volume was significantly decreased in subjects diagnosed for major depressive disorder (MDD) compared to age- and sex-matched healthy controls, using magnetic resonance imaging (MRI) and subsequent image size analysis (MacMaster and Kusumakar, 2004). Many research groups had already observed that rodents display adult neurogenesis in various regions of the brain, including the hippocampus (Gage *et al*, 1998), though it was thought to be a rodent-selective feature. Though, in other animal studies, researchers found that extended stress paradigms led to decreases in hippocampal volume, and could be reversed via antidepressant treatment, displaying parallels

to the human findings (Czéh *et al*, 2001; Murray *et al*, 2008). This line of research is largely influenced by a seminal study that showed that human hippocampal neurogenesis can occur into and through adulthood (Eriksson *et al*, 1998). Further studies have shown that antidepressant treatment stimulates the production of hippocampal neural progenitor cells, measured in post-mortem brain tissue of depressed individuals compared to both untreated depressed subjects and non-depressed untreated subjects (Boldrini *et al*, 2009). These results highlight interesting effects of chronic antidepressant administration, but they cannot indicate that hippocampal neurogenesis is required for antidepressant efficacy. In order to test for efficacy, studies were performed that both chronically dosed SSRIs and blocked hippocampal neurogenesis. When hippocampal neurogenesis was prevented via focal X-ray irradiation of hippocampal progenitor cells, the behavioral efficacy of chronic SSRI was blocked in the NSF assay (Santarelli *et al*, 2003). In this same study, the researchers also tested 5-HT_{1A} receptor KO mice—which are unresponsive behaviorally to chronic SSRI administration—and those mice did not exhibit the same increase in hippocampal neurogenesis as do WT mice under the same chronic SSRI administration paradigm (Santarelli *et al*, 2003). These data suggest that the 5-HT_{1A} receptor may be necessary for the SSRI mediated stimulation of hippocampal neurogenesis, as well as behavioral efficacy. Hippocampal neurogenesis may be required for SSRI behavioral efficacy, but do not seem to be required for the efficacy of tricyclic antidepressants, CRF-R antagonists, nor vasopressin antagonists (Santarelli *et al*, 2003; Surget *et al*, 2008). Altogether these findings provide the strongest evidence for a requirement of hippocampal neurogenesis for SSRI antidepressant behavioral efficacy, that SSRI action is mediated via 5-HT signaling, and can support the delay in SSRI clinical efficacy as arising from delayed neurogenic processes that occur on the same timescale as behavioral/clinical efficacy (van Praag *et al*, 2002).

It has also been shown that several antidepressant strategies ranging from exercise, pharmacological intervention, and electroshock therapy enhance hippocampal neurogenesis in the critical SGZ of the hippocampus (Malberg *et al*, 2000; van Praag *et al*, 1999; Scott *et al*, 2000), and do so through enhancement of division of neural progenitor cells (Encinas *et al*, 2006). Electroshock therapy and antidepressant treatments also potentiate the expression of brain-derived neurotrophic factor (BDNF) mRNA and protein in rat hippocampus (Altar *et al*, 2003; Russo-Neustadt *et al*, 2000). Peripheral administration of BDNF has also been shown to produce antidepressant effects in the novelty suppressed feeding assay (NSF), the forced swim task (FST), unpredictable chronic mild stress (UCMS), and in the elevated plus maze (EPM) (Schmidt and Duman, 2010). The BDNF receptor *trkB* neurotrophin receptor has been shown to be rapidly activated after acute administration of SSRI and seems to be required for behavioral efficacy (Saarelainen *et al*, 2003). These effects do not seem to be simply due to increasing cell number; chronic stress paradigms, like those used to induce a ‘depressed’ state in animals, show that while there is a decrease in hippocampal volume, the effect is not due to reduction in hippocampal cell number (Lucassen *et al*, 2001; Müller *et al*, 2001). Comparable findings showed that stress induces dendritic remodeling of CA3 pyramidal neurons, reduces the number of synapses on those neurons, and impairs neurogenesis in the dentate gyrus (Fuchs *et al*, 2006). Other studies have suggested similar mechanisms for their findings, namely that neurogenesis and dendritic spine growth could be involved in SSRI response (Costa e Silva, 2004; D’Sa and Duman, 2002). These findings strongly suggest a neurotrophic effect of SSRI efficacy (Castrén, 2004). Although the evidence is strong that hippocampal neurogenesis is required for SSRI antidepressant efficacy, further studies need to be performed to establish the pathway leading from increased 5-HT signaling to hippocampal neurogenesis.

Serotonergic Evidence of SSRI Efficacy by Direct Receptor Analysis

As noted above, mechanistic determination for the molecular actions of SERT antagonist based antidepressants began long prior to the molecular cloning of SERT. Through the analyses that drugs or treatments that reduced 5-HT tone could produce depressive-like effects and drugs and treatments that enhanced 5-HT tone could produce antidepressant-like effects helped solidify the role of 5-HT system disruption in depression pathology. Due to the serendipitous discovery of reuptake based antidepressants, primarily considering the serotonergic system and SERT, serotonin specific reuptake inhibitors (SSRIs) are the most general and means of ubiquitous enhancement of serotonergic signaling. This strategy, however, does not provide much direction to the specificity of the effects, and by which 5-HT receptor—or receptors—is the antidepressant effect of serotonergic signal enhancement enacted. It may be of great scientific interest to investigate which receptors, or set of receptors, is responsible for the antidepressant effect of SSRIs, as this determination could allow for more targeted approaches in the future that could bypass the delay in antidepressant clinical efficacy and/or improve the side effect profile of future antidepressant drugs.

Following sufficient SSRI induced SERT blockade, 5-HT levels elevate in the synapse and activate pre- and post-synaptic serotonin receptors. There are 7 main types of 5-HT receptors, of which there exist subfamilies within some types, totaling over 20 different kinds of 5-HT receptors in the mammalian system, not including RNA edited 5-HT receptors (Fitzgerald *et al*, 1999). All but one of these are 7-transmembrane G-protein coupled receptors (GPCRs), the exception being the 5-HT₃ receptor, which is a ligand gated ion channel (**Table 1**).

The 5-HT_{1A} receptor has received much attention with respect to the actions of SSRIs as it is the main autoreceptor for serotonergic neurons, regulating the firing rate of 5-HT neurons by negative feedback (Barnes and Sharp, 1999). 5-HT_{1A} receptors are coupled to G α i, which inhibits the activity of adenylate cyclase, thus acting as a negative regulator of synaptic firing and signal propagation via cAMP and PKA pathways. The delay in SSRI clinical efficacy is thought to be due to the counterproductive initial diminishing of serotonergic firing rate, as SSRI mediated SERT blockade and enhancement of post-synaptic 5-HT receptor signaling will activate 5-HT_{1A} autoreceptors and suppress the firing rate of serotonergic neurons, diminishing overall serotonergic signaling tone (Le Poul *et al*, 1995). Chronic and clinical SSRI efficacy is thought to be the result of, in part, 5-HT_{1A} desensitization, allowing these medications to potentiate 5-HT signaling following a recovery of normal firing rates. In support of this idea, recovery from 5-HT_{1A} receptor desensitization follows the timecourse of chronic SSRI efficacy (Le Poul *et al*, 1995). 5-HT_{1A} receptors also exist postsynaptically, where they act in regulatory manners on non-serotonergic neurons, and this may be involved with the SSRI response. 5-HT_{1A} receptor global knockout mice are behaviorally insensitive chronically to SSRIs and additionally do not display increased proliferation of hippocampal stem cells, a trait that is required for chronic SSRI behavioral efficacy (Santarelli *et al*, 2003). When targeted specifically with direct agonism at 5-HT_{1A}, WT animals display an antidepressive-like effect in the forced swim test (FST) (Detke *et al*, 1995; López-Rubalcava and Lucki, 2000; Robinson *et al*, 1990).

Receptor	Subtype	Coupling	Function	
5-HT1	1A	G α i/o	Autoreceptor: inhibitory on synaptic fusion	Inhibitory, PKA Pathways
	1B		Autoreceptor: inhibitory on synaptic fusion	Inhibitory, PKA Pathways
	1D		Inhibitory, PKA Pathways	
	1E			
	1F			
5-HT2	2A	G α q	Stimulatory, PKC Pathways	
	2B			
	2C			
5-HT3	-	Ligand Gated Ion Channel; Na ⁺ Permeant	Stimulatory, Neuroexcitable	
5-HT4	-	G α s	Stimulatory, PKA Pathways	
5-HT5	5A	G α i/o	Inhibitory, PKA Pathways	
	5B			
5-HT6	-	G α s	Stimulatory, PKA Pathways	
5-HT7	-	G α s	Stimulatory, PKA Pathways	

Table 1. Highlighted Major 5-HT Receptors. Table displays their major subtypes and G α couplings, if applicable, and the functional result of their activation.

Though, when a 5-HT_{1A} receptor antagonist was added to the SSRI fluoxetine, the decrease in immobility is enhanced in the FST (Detke *et al*, 1995). These findings suggest a complicated role of 5-HT_{1A} receptors in the antidepressant like effects of SSRIs, though this could be a result of various 5-HT_{1A} selective ligands having preference for pre- versus post-synaptic 5-HT_{1A} receptors with unknown mechanism (Lladó-Pelfort *et al*, 2010), which could explain the differential effects of these different ligands in various assays. Though different experiments yield conflicting results regarding the utility of 5-HT_{1A} in antidepressant efficacy and in the antidepressant activity of SSRIs, these data show the important role of the receptor in the ability to regulate antidepressant-like effects *in vivo*.

Another important receptor in the actions of SSRI actions is the 5-HT_{2C} receptor. The 5-HT_{2C} receptor is a GPCR coupled to the G α _q G-protein, which, upon 5-HT_{2C} receptor activation, activates PLC which cleaves PIP₂ to IP₃ (which stimulates the release of intracellular Ca²⁺ stores) and DAG (which activates PKC along with increased Ca²⁺ levels). 5-HT_{2C} receptor agonists, like SSRIs, induce significant decreases in immobility time in the FST (Cryan and Lucki, 2000). Importantly, 5-HT_{2C} receptor antagonists pretreatment blocked the immobility inducing effects of fluoxetine (Cryan and Lucki, 2000). Additionally, 5-HT₂ receptors are implicated for the regulatory role in hippocampal neurogenesis (Klempin *et al*, 2010). These studies show that 5-HT_{2C} signaling is critical for early phase SSRI antidepressant-like effects, and suggest that specific 5-HT_{2C} receptor agonists might serve as effective antidepressants on their own. 5-HT_{2C} could serve as a viable pharmacological target for future antidepressants, though it has largely been avoided due to the high homology to the 5-HT₂ receptor subtypes, specifically 5-HT_{2A}, which often has oppositional effects (Bressa *et al*, 1987), as well as the potential for untoward clinical effects due

to a role of 5-HT_{2A/2C} in the hallucinatory effects brought about by LSD, psilocybin, and DMT (Baumeister *et al*, 2014; Cussac *et al*, 2008).

Other studies have highlighted the role of 5-HT_{1B} in the actions of SSRIs. 5-HT_{1B}, like 5-HT_{1A}, is a G α i coupled GPCR. 5-HT_{1B} specific agonists have been shown to possess antidepressant-like effects in the FST (Tatarczyńska *et al*, 2005), and antagonists of 5-HT_{1B} receptor, block the SSRI induced decrease in immobility in the FST (Chenu *et al*, 2008).

5-HT₄ receptor agonism is also capable of producing antidepressant-like effects (Lucas *et al*, 2010). 5-HT₄ is a G α s coupled GPCR, that when activated, stimulates the activity of adenylate cyclase, which elevates levels of cAMP, activating PKA pathways and promoting signal propagation. Additionally, targeting other 5-HT receptors have been shown to be capable of producing antidepressant-like effects. Of note, antidepressant-like effects can be generated via antagonism of 5-HT_{2A} (Patel *et al*, 2004), 5-HT₃ (Ramamoorthy *et al*, 2008), and 5-HT₇ (Wesołowska *et al*, 2006) receptors.

These data reinforce the importance of serotonergic signaling in both antidepressant-like effects alone in addition to their role in SSRI produced antidepressant-like effects. These effects provide evidence that SSRIs mediate their effects through 5-HT receptors, albeit globally. Due to the antidepressant-like effects that are generated via blockade of 5-HT_{2A}, 5-HT₃, and 5-HT₇ receptors, engagement of these targets via global enhancement in serotonergic signaling via SSRI treatment may actually be deleterious to the desired antidepressant effect. In fact, agonists of 5-HT₃ receptor block the effect of SSRIs in the FST (Nakagawa *et al*, 1998). Future antidepressant drug development may aim to selectively target the desired 5-HT receptors and either avoid or antagonize these pro-depressive receptors for a better range of efficacy. In this aim, a newly approved antidepressant, vortioxetine, possesses activity at SERT (blocker), 5-HT_{1A} (agonist), 5-

HT_{1B} (partial agonist), 5-HT₃ (antagonist), and 5-HT₇ (antagonist), all in the aim of attaining faster clinical efficacy (Guilloux *et al*, 2013; Mørk *et al*, 2012). Vortioxetine is discussed in detail in Chapter 5, though this novel approach represents an approach to update serotonin based antidepressants using modern research directives catering to the multifaceted roles of serotonin in the brain.

SERT-Independent Targets of SSRIs

SSRIs, despite the eponymous claim to specificity, are sufficiently promiscuous in their off-target actions to warrant evaluation of other targets besides SERT (Bianchi, 2008; Owens *et al*, 1997). The prevailing thought deemed these off-target binding events are suggested to contribute towards SSRI side-effect profiles. At face value, this seems appropriate, but it is important to consider first how drug selectivity screens are generally performed. Most screens are limited to a select panel of targets due to cost limitations, and binding events with a K_D higher than 1 μ M are generally discarded (Bianchi, 2008). This practice is problematic when it comes to SSRIs in particular, because upon normal dosing paradigms, central nervous system (CNS) concentrations of SSRIs are estimated to range from 1-10 μ M (Karson *et al*, 1993). Taking into consideration this expanded window of potentially relevant binding events, many more targets should be considered as relevant to antidepressant mechanisms. SSRIs and their metabolites bind to—and modulate—a sizable number of non-trivial targets at physiologically relevant concentrations, which could be contributing to efficacy (Bianchi, 2008; Owens *et al*, 1997). These targets include multiple voltage gated ion channels, activation of GABA-A receptors, inhibition of

nicotinic acetylcholine receptors (nAChR), and activity at various serotonin receptors (Bianchi, 2008; Owens *et al*, 1997; Zhang *et al*, 2010).

One prominent example of the potential contribution towards efficacy is the inhibition of TREK-1 channel, which is a widely expressed potassium channel. These channels contribute to the leak current, are regulatable, and are heavily expressed in areas relevant to antidepressant mechanisms, including hippocampus, cortex, striatum, amygdala, and hypothalamus (Heurteaux *et al*, 2006). It has been found that fluoxetine (and its metabolite norfluoxetine), sertraline, and paroxetine all inhibit the TREK-1 channel and have IC₅₀'s well within the predicted brain concentration for SSRIs (Bianchi, 2008). TREK-1 KO mice display a basal antidepressive phenotype, and appear behaviorally insensitive to SSRIs (Heurteaux *et al*, 2006). Interestingly, these mice still exhibited an enhancement in hippocampal neurogenesis, and displayed even stronger SSRI potentiated hippocampal neurogenesis than WT control mice (Heurteaux *et al*, 2006).

It has also been shown that certain SSRIs can activate σ -1 receptors, and that directed activation of the σ -1 receptor is sufficient for antidepressant-like actions, with effects blocked by specific σ -1 receptor antagonists, and are absent in σ -1 receptor KO mice (Villard *et al*, 2011; Wang *et al*, 2007). Furthermore, σ -1 receptor activation can potentiate NGF driven neurite outgrowth, effects that are prevented with specific σ -1 receptor antagonists (Nishimura *et al*, 2008). On this note, the SSRI citalopram induces neurite outgrowth in embryonic thalamic explants, effects due to its activation of the σ -1 receptor (Bonnin *et al*, 2012). The growth promoting effects of citalopram can be blocked by a σ -1 receptor antagonist, and are retained in a citalopram insensitive mouse line (SERT Met172) (Bonnin *et al*, 2012).

The SSRI sertraline has been shown to be biologically active in yeast, despite the fact that yeast do not express any version of SERT (Rainey *et al*, 2010). Furthermore that sertraline was able to inhibit PLA₁, PLC, and PLD while stimulating PLA₂ *in vitro* (Rainey *et al*, 2010). PLA₂ has been implicated in the antidepressant effects of maprotiline (a norepinephrine reuptake inhibitor; NRI) (Lee *et al*, 2011), and PLC_β & PLC_γ inhibition produces an antidepressant effect in the forced swim task (FST) (Galeotti and Ghelardini, 2011). Sertraline has also been shown to activate the glucocorticoid receptor and lead to hippocampal neurogenesis, and that this effect could be blocked with a glucocorticoid receptor antagonist (Anacker *et al*, 2011).

SSRIs also have activity directly at 5-HT receptors (Owens *et al*, 1997; Zhang *et al*, 2010), which, when these 5-HT receptors are stimulated directly, can produce antidepressant-like effects on their own. If SSRIs can produce antidepressant effects directly through their actions at 5-HT receptors, then SERT might not be required for their activity. Additionally, certain SSRIs have activity at alpha-1 and alpha-2 receptors (Owens *et al*, 1997). These receptors are norepinephrine receptors, and though not the focus of this document, norepinephrine signal enhancement is sufficient to produce antidepressant effects (Lee *et al*, 2011; Santarelli *et al*, 2003; Sulser, 1984), like serotonin, though serotonin has garnered the majority of the research focus due to the biological links to the chemical and the clinical presentation of depression.

Certain SSRIs display high affinity for muscarinic acetylcholine receptors (mAChR) (Owens *et al*, 1997; Snyder and Yamamura, 1977) as well as the nicotinic acetylcholine receptor (nAChR) (García-Colunga *et al*, 1997). Nicotinic acetylcholine receptors have been proposed to be a new target for antidepressant development, as their targeting can produce antidepressant-like behavioral effects (Shytle *et al*, 2002). Scopolamine, a muscarinic receptor agonist, has also been

demonstrated to have antidepressant activity, particularly for the rapid treatment of depression (Drevets *et al*, 2013).

As noted above, there is significant crossover of antidepressant effects from multiple monoaminergic systems. Selective norepinephrine reuptake inhibitors (eg. desipramine) and agents that target dopamine receptors (eg. pramipexole) exist as clinically viable antidepressants in their own right, as well as the importance of the mesolimbic dopaminergic system in depression pathophysiology (Nestler and Carlezon, 2006). On this note, it is important to illuminate that these monoaminergic reuptake proteins have significant substrate affinity crossover, leading to situations whereby SERT can feasibly uptake dopamine, where it can be packaged alongside 5-HT in vesicles (Larsen *et al*, 2011), as well as the potential for 5-HT uptake via dopamine and/or norepinephrine transporter (DAT; NET) (Daws, 2009). The SSRI fluoxetine, at the high doses required for clinical efficacy, can also induce elevations in norepinephrine and dopamine levels (Koch *et al*, 2002). The SSRIs paroxetine, sertraline, and fluoxetine all possess moderate affinity for NET and DAT, indicating a potential role for non-SERT effects of their actions supporting antidepressant mechanisms (Owens *et al*, 2001).

These findings described above raise an interesting possibility, namely that these off-target effects of SSRIs may contribute to, or drive antidepressant efficacy of SSRIs. It is not known how important these effects are to the overall efficacy of SSRIs, since it has been impossible to separate the 5-HT based mechanisms of SSRIs from these off-target effects. Alternatively, these non-SERT actions of SSRIs could be contributing to the extent or magnitude of effects driven by SERT antagonism.

The SERT Met172 Mouse Model for Measuring 5-HT Dependence

In order to distinguish off-target effects from 5-HT dependent effects, initial attempts utilized mice possessing a genetic deletion of the serotonin transporter gene (SERT KO). Although a seemingly appealing strategy, these animals display major compensatory alterations that limit their utility as an animal model (Baganz *et al*, 2008; Kalueff *et al*, 2010; Murphy and Lesch, 2008). These SERT KO animals display a dramatic reduction in brain 5-HT levels (Bengel *et al*, 1998), 5-HT neurons (Lira *et al*, 2003), expression of various 5-HT receptors (Cour *et al*, 2001; Fabre *et al*, 2000; Li *et al*, 2000; Rioux *et al*, 1999), and changes dorsal raphe firing rate (Lira *et al*, 2003). These changes also impact basal behavior; SERT KO mice display spontaneous ‘depressed’ phenotypes in various behavioral assays (Lira *et al*, 2003), including an unusual basal ‘learned helplessness’ phenotype whereby SERT KO mice require no training to produce escape failures in the shuttlebox assay, whereas WT mice do require training (Caldarone *et al*, 2000; Vaugeois *et al*, 1996). These aberrations in normal neurobiology and behavior make it extremely difficult to link the subtle effects of SSRIs and extrapolate to the context of normal physiology of serotonin neurons and their synapses.

Our lab realized that to pursue the separation of 5-HT and off-target effects of SERT blockers, including SSRIs, a mouse model was needed that could reduce SSRI interactions at SERT without any disruptions of normal physiology or the serotonergic network. Such a model essentially required disruption of SERT binding for SERT targeted drugs, but not for 5-HT. It has been observed that human and *Drosophila melanogaster* SERT (hSERT and dSERT, respectively) display markedly different sensitivities to most SSRIs while having similar 5-HT transport abilities. Through a careful set of mutagenesis experiments, our lab discovered that the species

dependence of SSRI sensitivity could be attributed to variation at a single amino acid residue in the inner pore structure of the transporter proximal to the predicted 5-HT binding site (I172 in human and mouse, M167 in fly) (Henry *et al*, 2006) (**Figure 5**) (**Figure 6**). Mutating I172 in hSERT and mSERT to Met172 could recapitulate the dramatic reduction in inhibition efficacy for many SERT antagonists as is seen in dSERT, without affecting 5-HT transport (Henry *et al*, 2006). Interestingly, paroxetine displays highly potent inhibition of dSERT as well as hSERT and mSERT, and is unaffected by the I172M substitution (Henry *et al*, 2006). Additionally, mutating dSERT to the respective mammalian amino acid (M167I) produces a gain in SSRI potency (Henry *et al*, 2006). With this knowledge, our lab then created a mouse with the I172M substitution in SERT (Thompson *et al*, 2011). These mice displayed normal growth, normal SERT expression, and no overt morphological differences or basal phenotype (Thompson *et al*, 2011). The mice also displayed normal 5-HT uptake, 5-HT clearance rate, and 5-HT induced reductions in raphe firing rates (Thompson *et al*, 2011). As expected, *ex vivo* synaptosomal analyses revealed that this mutation rendered dramatic reductions in inhibition efficacy of [³H]5-HT uptake for many SERT blockers (Thompson *et al*, 2011). Consequently, these SERT Met172 mice do not exhibit normal increases in extracellular 5-HT after citalopram or fluoxetine administration (20 mg/kg, i.p.) as measured by *in vivo* microdialysis, displayed no reduction in dorsal raphe firing rate after administration of 1 μ M citalopram in *ex vivo* slice recordings, and displayed no alterations in 5-HT clearance rate after administration of citalopram and fluoxetine (Thompson *et al*, 2011). Next, our lab assayed for behavioral sensitivity to SSRIs in the forced swim task (FST) and the tail suspension test (TST), which have predictive validity for monoaminergic antidepressants and are sensitive to acute antidepressant administration (Lucki *et al*, 1994). The results of these studies were not completely clear.

		TM3																														
human	157	I	F	K	G	I	G	Y	A	I	C	I	I	A	F	Y	I	A	S	Y	Y	N	T	I	M	A	W	A	L	Y	Y	L
ram	157	I	F	K	G	I	G	C	A	I	C	L	I	A	F	Y	I	A	S	Y	Y	N	T	I	M	A	W	A	L	Y	Y	L
sheep	157	I	F	K	G	I	G	C	A	I	C	L	I	A	F	Y	I	A	S	Y	Y	N	T	I	M	A	W	A	L	Y	Y	L
bovine	157	I	F	K	G	I	G	C	A	I	C	L	I	A	F	Y	I	A	S	Y	Y	N	T	I	I	A	W	A	L	Y	Y	L
macaque	157	I	F	K	G	I	G	Y	A	I	C	I	I	A	F	Y	I	A	S	Y	Y	N	T	I	M	A	W	A	L	Y	Y	L
cat	157	I	F	K	G	I	G	Y	A	I	C	I	I	A	F	Y	I	A	S	Y	Y	N	T	I	M	A	W	A	L	Y	Y	L
rat	157	I	F	K	G	I	G	Y	A	I	C	I	I	A	F	Y	I	A	S	Y	Y	N	T	I	I	A	W	A	L	Y	Y	L
mouse	157	I	F	K	G	I	G	Y	A	I	C	I	I	A	F	Y	I	A	S	Y	Y	N	T	I	I	A	W	A	L	Y	Y	L
guinea pig	157	I	F	K	G	I	G	Y	T	I	C	I	I	A	F	Y	I	A	S	Y	Y	N	T	I	I	A	W	A	L	Y	Y	L
drosophila	111	A	L	K	G	V	G	Y	A	I	C	L	I	D	I	Y	M	G	M	Y	Y	N	T	I	I	G	W	A	V	Y	Y	L
mosquito	092	A	L	K	G	V	G	Y	A	I	C	L	I	D	I	Y	M	G	M	Y	Y	N	T	I	I	G	W	A	V	Y	Y	L
tobacco moth	116	A	L	K	G	V	G	Y	A	I	C	M	I	D	I	Y	M	G	M	Y	Y	N	T	I	I	G	W	A	V	Y	Y	L

Modified from: Henry, L. K. et al. *J. Biol. Chem.* 2006;281:2012-2023

Figure 5. Sequence Alignment of SERT Protein. Linear sequence alignment of various SERT proteins across phylogeny, highlighting transmembrane 3 domain of SERT. Membrane primary structure displays high sequence conservation across this transmembrane region. Highlighted is a prominent example of a sequence variant seen in drosophila at position 172. The drosophila SERT, while displaying capable uptake of 5-HT, it displays disrupted affinity for many SERT blockers. This lowered affinity can be attributed to this single amino acid substitution Ile172Met.

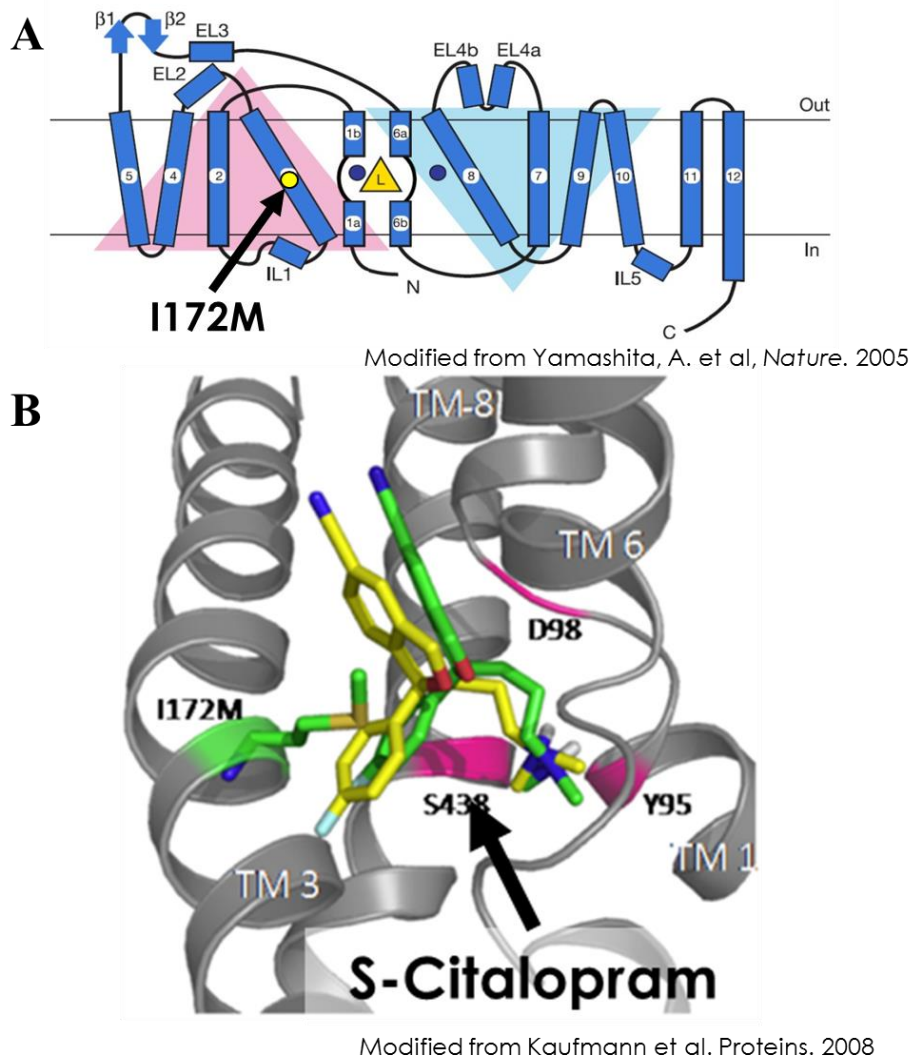


Figure 6. Structure of SERT with I172M Substitution. **A.** 2D Structure of SERT, Based upon LeuT Structure. Two dimensional homology model mapping of SERT, based upon the LeuT crystal structure. These data align with previous predictions made upon the cloning of the rodent SERT, that the protein contains 12 transmembrane domains. Highlighted is the position of amino acid 172, and the position of the Ile172Met substitution, derived from the dSERT studies. When this flattened model is remapped onto three dimensional space, Ile172 is positioned directly adjacent to the predicted ligand (both SSRI and 5-HT) binding site (“L” triangle, above), and required for high affinity binding of SERT blockers at SERT. The drosophila SERT, where our model intellectually originated, while displaying capable uptake of 5-HT, it displays disrupted affinity for many SERT blockers. This lowered affinity can be attributed to this single amino acid substitution Ile172Met. **B.** 3D homology model of hSERT with computational binding of s-citalopram. The I172M substitution perturbs structural binding potential for s-citalopram in hSERT, predicted to reduce affinity for the drug for SERT Met172

Whereas the SERT Met172 mice displayed a lower change in immobility in the TST compared to WT animals following treatment with the SSRIs citalopram and fluoxetine, these results did not attain significance (Thompson *et al*, 2011). Furthermore, when tested in the FST, WT animals responded with significant increases in immobility following treatment with SSRIs, but the SERT Met172 mice were insensitive to SSRI treatment of citalopram and fluoxetine, but remained sensitive to paroxetine (Thompson *et al*, 2011). We suspected that the behavioral assays were hampered by to the genetic background of the mouse. The original SERT Met172 line was generated using 129S6 derived embryonic stem cells (ES cells). It has been reported that mice with a 129 genetic background have poor response to SSRIs compared to other mouse and rat models, and that a C57BL/6 background would be more suitable for behavioral assays of this nature (Crowley *et al*, 2005; Gingrich and Hen, 2000; Jacobson and Cryan, 2007; Lucki *et al*, 2001).

Rationale for Thesis Studies

Although there is much support for a 5-HT link to depression and the actions of SSRIs, there are reasons to be cautious as to our attribution of drug actions *in vivo* as solely linked to alterations in 5-HT signaling. Most troubling is the well-known issue that SSRIs are not immediately efficacious, even though these drugs block SERT within minutes. This observation indicates that time-dependent plasticities such as changes in membrane excitability, synaptic morphology and/or stem cell production are needed to achieve therapeutic benefit, though it is not clear whether these changes all derive from SERT-dependent drug targeting. In this regard, many proteins encoded in the human genome do not have assays that can be used to define, holistically,

the nature of drug specificity. Even within the “druggable genome” SSRIs have been found to interact with a sizable number of non-trivial targets at physiologically relevant concentrations. These targets, when manipulated specifically, can induce some of the biochemical and behavioral effects of both acute and chronic SSRI administration, raising the question as to whether all of the effects of SSRIs are 5-HT mediated. In order to separate the 5-HT/SERT-dependent effects of SSRIs from their non-5-HT/SERT/independent effects, I utilize a novel transgenic mouse strain developed by the Blakely lab wherein a point mutation has been introduced in *Slc6a4* to convert an Ile at amino acid 172 to Met (Ile172Met; I172M), a substitution that confers insensitivity to many SERT blocking drugs both *in vitro* and *in vivo*. Critically, the I172M transgenic model displays normal levels of SERT protein and 5-HT clearance, eliminating concerns of compensatory changes in 5-HT homeostasis and signaling physiology that attend many other transgenic models. Using the SERT Met172 model backcrossed to the C57Bl/6 background, I sought to establish whether SERT blockade *in vivo* is required for both acute and chronic SSRI-modulated behavior and biochemistry. These efforts provide the most definitive analysis to date of the relationship of 5-HT signaling to SSRI action *in vivo*. To achieve these goals, I pursued the following Specific Aims:

Ascertain the Biochemical Inconsequentiality of Backcrossing the SERT Met172 Mouse onto the C57Bl/6 Genetic Background:

Due to the necessity to backcross the SERT Met172 mouse model onto a more suitable genetic background for further behavioral assessment, I first test to ensure that the act of backcrossing the SERT Met172 model from the 129S6/S4 hybrid background onto the C57Bl/6

genetic background has not somehow negatively impacted aspects that would impede my efforts in planned further studies, such as differential gene regulation impacting SERT Met172 expression. I test whether backcrossing has impacted SERT protein expression, SERT protein uptake function, biogenic amine concentrations, and lastly whether the backcrossing has impacted our expected shifts in pharmacology for fluoxetine and citalopram, and preservation of sensitivity to paroxetine.

Determine Whether Acute Behavioral Sensitivity to SSRIs Requires SERT Blockade:

Using adult, male SERT Met172 mice congenic on a C57BL/6J background, I examine the actions of SSRIs that either are or are not influenced by the I172M substitution using behavioral assays known to be sensitive to acute SSRI treatment. These assays are known to possess predictive validity for antidepressants utilized in the clinic, and have been well characterized by antidepressant researchers for acute antidepressant drug sensitivity. It is with these assays that many off-target/non-SERT targets of SSRIs have been tested and claim non-SERT antidepressant effects of SSRIs, rendering them most suitable to test the requirement of SERT for the acute antidepressant-like actions of SSRIs

Determine Whether Chronic Behavioral and Biochemical Sensitivity to SSRIs Requires SERT Antagonism:

As with the studies testing whether acute behavioral sensitivity to SSRIs require SERT antagonism, I examine the SSRI sensitivity of SERT Met172 mice following chronic

administration of SSRIs in a model that is sensitive to chronic—but not acute—administration of SSRI. The assays chosen, novelty induced hypophagia and hippocampal neurogenesis, are thought to probe different facets of SSRI efficacy, and through their use, I will seek to establish the SERT-dependence of SSRI actions acutely and chronically. I will also investigate biochemical processes that are required for behavioral efficacy in this chronic behavioral antidepressant sensitivity model. Increased hippocampal stem cell proliferation and/or survival have been proposed to be critical for the actions of antidepressants in some, but not all, behavioral changes arising from chronic SSRI administration. In this Aim, I will employ BrdU-based methods to determine whether changes in stem cell generation and/or survival following SSRI administration require SERT blockade.

Evaluate the Requirement of SERT Antagonism for the Novel Multimodal Antidepressant Vortioxetine:

Utilizing the above assays, I test whether the removal of SERT antagonism from the pharmacological profile of vortioxetine, through the use of the SERT Met172 mouse model, would result in preserved antidepressant-like activity in assays that are sensitive to acute and chronic antidepressant administration. Such findings, regardless of outcome, would illuminate the necessity of SERT for future serotonergic antidepressants.

CHAPTER II

BIOCHEMICAL CHARACTERIZATION OF SERT MET172

Note: The work presented in this chapter was published as: Nackenoff AG, Moussa-Tooks AB, McMeekin AM, Veenstra-VanderWeele J, Blakely RD (2015). Essential Contributions of Serotonin Transporter Inhibition to the Acute and Chronic Actions of Fluoxetine and Citalopram in the SERT Met172 Mouse. *Neuropsychopharmacology*

Introduction

The initial studies on the SERT Met172 animals were performed upon a mouse line of the genetic background of 129 lineage. From *in vitro* and *in vivo* studies, these prior studies were able to demonstrate and recapitulate prior hypotheses that the SERT I172M substitution yields a SERT completely able to be expressed, trafficked to the plasma membrane, and to uptake 5-HT with normal kinetics while rendering SERT Met172 incapable of being high affinity interactions with most SERT antagonists. The initial mouse line onto which the SERT Met172 substitution was introduced via knock-in techniques, however, was observed to exhibit low spontaneous activity in many behavioral tasks, low basal anxiety, and poor behavioral sensitivity to neuropsychiatric drugs (Jacobson and Cryan, 2007), which limits their utility in studies that rely upon behavioral screening endpoints to evaluate the specificity of SSRI action. It was for this reason that we sought to improve upon previous studies (Thompson *et al*, 2011), which required further manipulations in order to assess our hypothesis regarding the ablation of SSRI behavioral sensitivity rendered by the SERT Met172 substitution. It was therefore necessary to mobilize this genetic substitution onto a genetic background that would be more conducive to baseline anxiogenic behavior and

behavioral drug sensitivity studies. In these aims, we backcrossed the original 192S6/S4 line onto the C57Bl/6 genetic background, as described in detail below.

The backcross, simply put, aims to preserve the SERT gene locus from the engineered 129 mouse line, but replace the rest of the genome with C57Bl/6 derived DNA. The theory here is that any and all genes that would render the 129 mouse useless in behavioral tests are replaced by the C57Bl/6 versions of these genes. A major consideration, however, is that we cannot know all the interplay of physically interacting and regulatory proteins and kinases that interact with SERT that would also be altered in this process. In other systems, notably 7-transmembrane G-protein coupled receptors (GPCRs), there are proteins that regulate the pharmacological sensitivity to endogenous and exogenous ligands (eg: Han *et al*, 2010), which, if their expression or function were altered through protein expression level or through phylogenetic sequence differences, would alter the perception of GPCR pharmacology between mouse strains, though we would potentially be blind to the mechanism. SERT is also known to be regulated by a number of kinases, and the magnitude of SERT regulation may differ across different mouse strains (Carneiro *et al*, 2009; Veenstra-VanderWeele *et al*, 2012; Ye and Blakely, 2011). We must therefore consider that backcrossing the SERT Met172 mice onto the C57Bl/6 genetic background could have unintended consequences pertaining towards SERT Met172 sensitivity to SSRIs or to its affinity and trafficking potential for 5-HT. Failure to do so could confound future conclusions pertaining to the I172M substitution were the backcrossing onto the C57Bl/6 genetic background be to blame.

Therefore, following the successful backcrossing of both the wildtype SERT Ile172 and knock-in SERT Met172 allele onto the C57Bl/6 background, I tested important facets of *ex vivo* SERT function to confirm the biochemical findings performed previously. Based upon these results, I tested—and expected to find—that the backcrossing onto the C57Bl/6 background did

not alter total SERT protein expression, biogenic amine homeostasis, SERT specific 5-HT uptake. Importantly, this backcrossing did not impact the reduced affinity for the SSRIs fluoxetine and citalopram, while maintaining equipotent inhibition of the SSRI paroxetine.

Materials

Animals

SERT Met172 knock-in mice on a 129S6/S4 background were created as described previously (Thompson *et al*, 2011). To move to a background more conducive for the study of SSRI sensitivity (Crowley *et al*, 2005; Gingrich and Hen, 2000; Jacobson and Cryan, 2007; Lucki *et al*, 2001), we backcrossed animals until the SERT Met172 allele was >99% congenic onto a C57BL/6J background using a single nucleotide polymorphism-based speed congenic approach (Jackson Labs, Bar Harbor, Maine, USA). Because the SERT gene (*Slc6a4*) in the 129S6 embryonic stem cells used to generate the SERT Met172 knock-in line harbors a functional, two amino acid difference with the C57BL/6J SERT (129: E³⁹R¹⁵² haplotype; C57: G³⁹K¹⁵² haplotype; Carneiro *et al*, 2009), it was also necessary to establish a second backcrossed line where the wildtype 129S6 *Slc6a4* gene was expressed on a C57BL/6J background, also established as >99% congenic. A cross of the latter line with the C57BL6/J SERT Met172 mice yields mice heterozygous for either SERT Ile172 or SERT Met172 (SERT^{I172/Met172}) with both *Slc6a4* alleles sharing the E³⁹R¹⁵² haplotype. These heterozygous mice were the parental animals for the homozygous SERT Ile172 (wildtype; WT) and SERT Met172 mice used in our studies. Initial characterizations of SERT levels, activity, and monoamine homeostasis were performed upon homozygous mice generated from heterozygous breedings. Because SERT levels, 5-HT clearance,

raphe 5-HT neuron firing rates, growth rates, and basal TST and FST behaviors were found to be equivalent in heterozygous bred SERT Ile172 and Met172 animals on both the 129S6/S4 (Thompson *et al*, 2011) and C57BL/6J backgrounds (these studies), we performed chronic drug treatment studies using WT and SERT Met172 mice generated from homozygous breedings. All animal studies were performed in accordance with protocols approved by the Vanderbilt University Animal Care and Use Committee.

Drugs

All general biochemical reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted, and were of the highest grade available. Fluoxetine HCl, citalopram HBr, and paroxetine HCl were acquired from TCI Chemicals (Portland, OR, USA). For *ex vivo* analyses, all drugs were prepared as 10mM stock solutions in 100% DMSO. Final DMSO concentrations for SSRI studies were under 1%. All control conditions contained the highest level of DMSO that would be presented by the most concentrated SSRI solution. Prior to i.p. injections and subsequent acute behavioral studies, drugs were prepared fresh in 0.9% sterile saline and filtered for sterility (Nalgene syringe filter, 0.2 μ m, 195-2520, ThermoFisher Scientific; Waltham, MA, USA).

SERT Western Blotting Analysis

Method

Following sacrifice by rapid decapitation, brain tissue from 8 week old male mice from midbrain, hippocampus, and frontal cortex (without olfactory bulb) was dissected and subjected to western blotting analysis for SERT protein. Samples were resolved on 10% gels by SDS-PAGE,

gels were transferred overnight to PVDF membrane (Immobilon, IPVH00010, Millipore) and then SERT was probed using guinea pig anti-SERT primary antibody (Frontier, 5HTT-GP-Af1400, 1:1000; Japan; 24 hrs at 4°C) and rabbit anti-guinea pig (1:10000; A-5545, Sigma; 1 hr at room temperature) as secondary antibody. Bands were detected by enhanced chemiluminescence (Clarity™ ECL substrate, Bio-Rad, Hercules, CA, USA). Membranes were stripped and loading was normalized after probing for mouse anti-β-actin (Sigma-Aldrich, A5441, 1:10,000; 1 hr at room temperature) and goat anti-mouse secondary antibody (1:10000; 115-035-062, Jackson ImmunoResearch, West Grove, PA, USA; 1 hr at room temperature). Blots were imaged and band density was quantified using ImageJ (<http://imagej.nih.gov/ij/>). For accurate comparisons, WT and SERT Met172 samples were intercalated, and only samples from one brain region were contained on each blot. Values at each brain region were compared using Student's t test, with alpha stringency set at $P = 0.05$.

Results

We sought to confirm that the SERT Met172 mutation did not alter SERT protein expression when expressed on a C57BL/6J background. Indeed, quantitative western blot analysis of SERT protein expression revealed no differences in frontal cortex, hippocampal, or midbrain SERT protein levels between WT and SERT Met172 mice (**Figure 7**). These data indicate a lack of consequential effects of the SERT Met172 mutant on basal SERT expression, aligning with prior studies (Henry *et al*, 2006; Thompson *et al*, 2011). Additionally, this confirms that there are no consequences from the act of backcrossing the SERT Met172 locus onto the C57Bl/6 genetic background with respect to the ability to express the SERT protein.

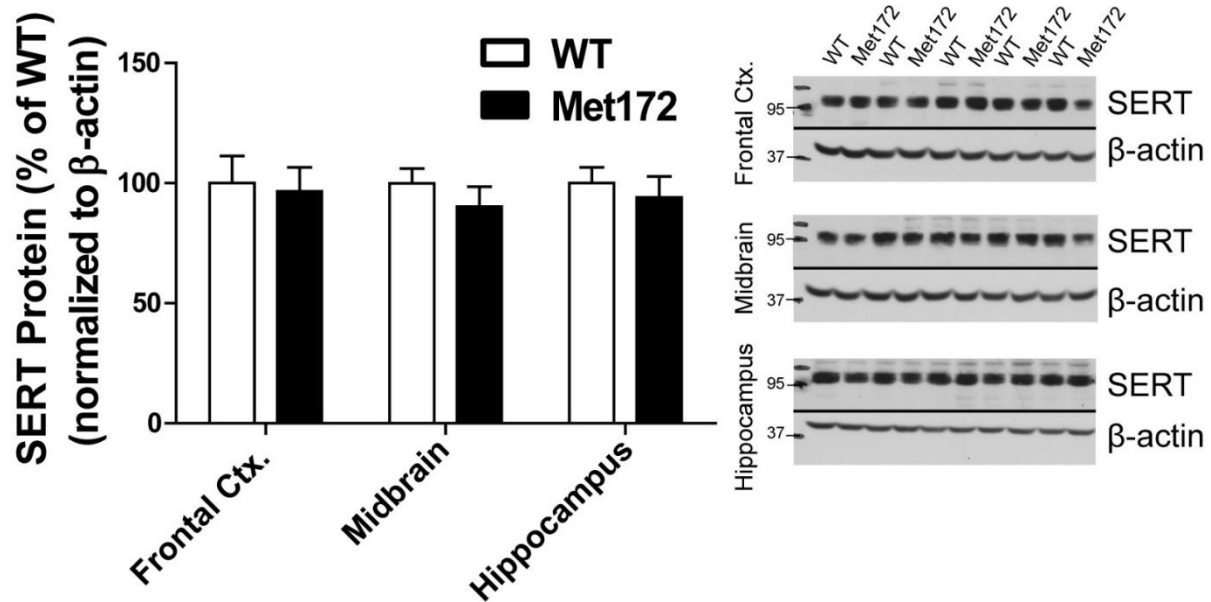


Figure 7. SERT Protein Expression in SERT Met172. The SERT Met172 mutant imposes altered pharmacological sensitivity with normal SERT expression and function. SERT protein expression was measured via Western blotting (left), and normalized to β -actin levels (right). SERT expression did not differ between WT and Met172 mice (two-tailed Student *t* test, $P > 0.05$; $n = 5$ per genotype and condition).

While we kept a close tab via PCR and strainotyping with respect to the coding sequence of SERT during the speed congenic approach to the backcrossing, there was still a possibility that regulatory elements and SERT gene promotor regions could be disrupted, for example, creating a situation where C57Bl/6 gene promotors would be less able to express SERT Met172 protein. These data indicate that our backcrossing efforts did not disrupt the ability of our hybrid mice to express either WT or SERT Met172.

Brain Monoamine Steady State Composition

Method

Male mice (8 weeks old) were sacrificed by rapid decapitation and brain regions of frontal cortex, hippocampus, and midbrain were dissected on ice and then rapidly frozen in test tubes on ethanol/dry ice and stored at -80 °C until extraction for assessment of monoamine neurotransmitters and metabolites. Monoamine levels were determined by HPLC/EC methods as described previously (Thompson *et al*, 2011) in the Vanderbilt Brain Institute Neurochemistry Core. Briefly, tissue samples were subjected to organic extraction in order to solubilize whole tissue neurotransmitters and their metabolites. The aqueous fraction containing these monoamines are forced through a chromatographic column via high pressure injection. This column separates the various monoamines via size and polarity and exit the column at different times. At this point, sample fractions are exposed to an electrochemical detector, and given a certain voltage oxidizes the substances in the sample based upon their redox potential. Sample oxidation generates a small electrical current, that, when compared to known concentrations of monoamines, can allow back calculation to determine exact concentration of the monoamine in the sample. Metabolite levels

were normalized to tissue extract protein concentration (Pierce BCA, #23225; ThermoFisher Scientific). Values at each brain region were compared using Students t test, with Bonferroni repeated measure correction, with alpha stringency set at $P = 0.05$.

Results

After organic extraction of neurotransmitters and their metabolites and subsequent chromatographic separation and electrochemical detection, we could determine that monoamine neurotransmitter and metabolite levels did not differ between genotypes across all brain regions (**Table 2**).

Saturation Uptake Kinetic Analysis of SERT

Method

Following sacrifice via rapid decapitation, the whole brains of 8-12 week old male mice were dissected on ice and used for synaptosomal preparations as described previously (Thompson *et al*, 2011). Briefly, midbrain sections were mechanically homogenized in 0.32M sucrose in 5mM HEPES buffer. This homogenate was subjected to a low gravity centrifugation (10 min, 4°C at 1,000x g) in order to remove large cellular components (eg. mitochondria, nuclei, and large membranes) from the reconstituted nerve terminals, classified as 'synaptosomes'. The supernatant after this first spin contains the synaptosomes. The synaptosomes are then pelleted via a high gravity centrifugation (15 min, 4°C at 10,000x g).

Substance	Frontal Cortex		Midbrain		Hippocampus	
	WT	Met172	WT	Met172	WT	Met172
5-HT	7.18±0.30	7.50±0.18	16.75±1.16	17.51±0.43	8.11±0.37	7.90±0.46
Dopamine	2.93±1.90	3.02±1.19	2.20±0.15	2.48±0.11	0.28±0.05	0.33±0.07
Norepinephrine	5.08±0.14	5.81±0.18	8.92±0.41	10.03±0.40	6.07±0.40	6.46±0.21
5-HIAA	2.86±0.17	2.88±0.22	11.76±0.97	11.25±0.58	5.14±0.34	4.37±0.30
DOPAC	0.81±0.19	0.82±0.17	1.08±0.08	1.09±0.04	0.19±0.03	0.16±0.01

Table 2. Biogenic Amines in SERT Met172. Values presented as mean ± SEM (ng/mg protein). Tissue levels were determined by HPLC-EC analyses as noted in Methods. Tissue levels of 5-HT, 5-HIAA, DA, DOPAC, and NE did not differ between WT and Met172 mice (n= 4 per condition, two-tailed Student's t test).

The pellet was resuspended in KRH buffer containing glucose (for synaptosomal viability throughout the preparation and assay), pargyline (MAOI, prevents enzymatic metabolism of 5-HT), and ascorbic acid (preservative, prevents spontaneous oxidation of 5-HT). Equal volumes of synaptosomes were then incubated at 37°C for 5 minutes with serial dilutions of 5-HT stock containing 10% [³H]5-HT (PerkinElmer, NET498001MC, Waltham, MA, USA). At each concentration of 5-HT, parallel samples were incubated in the presence of 1µM paroxetine (an SSRI), defining non-specific uptake (any residual radioactivity in these samples would be due to non-SERT uptake, passive diffusion, or indicate washing stringency), which was subtracted from total counts to yield specific uptake. 5-HT uptake into synaptosomes was terminated via addition of ice cold PBS buffer, and vacuum filtration through 0.3% polyethyleneimine treated glass fiber filters (GF/B, Whatman, Pittsburgh, PA, USA). Synaptosomes were assessed for protein concentration for normalization of 5-HT levels across experiments (Pierce BCA; ThermoFisher). Data were fit to rectangular hyperbolic Michaelis-Menten Kinetic regression curve fits (Graphpad Prism 6.0). Kinetic parameters (K_m and V_{max}) were compared using Student's t test, with alpha stringency set at $P = 0.05$.

Results

Raw values of radioactive uptake were converted into moles, via corrections for scintillation counting efficiency (using known concentrations of radioactivity), non-specific uptake, and the total time allotted in the uptake experiment. These data were fit to the amount of 5-HT added to the tubes, and fit to a Michaelis-Menten rectangular hyperbola to determine the kinetic parameters K_m and V_{max} (Graphpad Prism). We found no genotype effects on *ex vivo* 5-HT

transport kinetics in whole brain synaptosomes, neither in 5-HT K_m or 5-HT transport V_{max} ($P > 0.05$) (**Figure 8**).

Synaptosomal Competition Uptake Analysis

Method

Following sacrifice via rapid decapitation, the midbrains of 8-12 week old male mice were dissected on ice and used for synaptosomal preparations as described above, and previously (Thompson *et al*, 2011). For competition uptake assays, equal volumes of synaptosomes were incubated with 20 nM [3 H]5-HT (PerkinElmer) and varying concentrations of inhibitors and incubated at 37°C for 10 minutes, followed by vacuum filtration and scintillation spectroscopy methodology as described above. Data were fit to single site competition regression curve fit (Graphpad Prism 6.0).

Results

After confirming normal SERT expression and activity, we sought to verify whether the C57BL/6J backcrossed SERT Met172 allele maintained its reduced sensitivity to specific SERT antagonists (Henry *et al*, 2006; Thompson *et al*, 2011). To accomplish this, we performed competitive [3 H]5-HT uptake using midbrain derived synaptosomes of WT and SERT Met172 mice (**Figure 9**). These studies revealed the expected reductions in potency for fluoxetine (~20 fold) and citalopram (~500 fold) at SERT Met172.

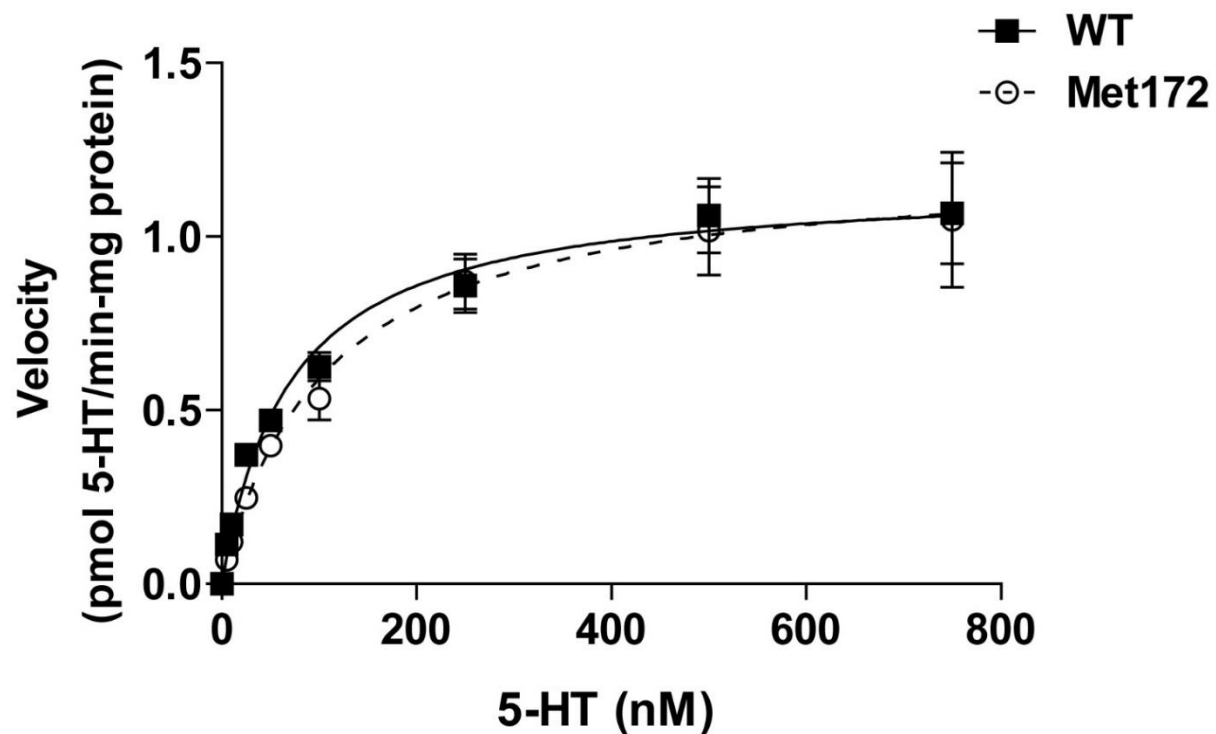


Figure 8. Saturation Uptake Kinetics. The SERT Met172 mutant imposes altered pharmacological sensitivity with normal SERT expression and function. Saturation uptake kinetics in WT and SERT Met172 whole brain synaptosomes. WT and Met172 do not differ in 5-HT transport activity (WT: K_m 73.04 ± 15.66 nM; V_{max} 1.18 ± 0.07 pmol/min-mg protein; Met172: K_m 102.0 ± 24.61 nM; V_{max} 1.22 ± 0.09 pmol/min-mg protein: two-tailed unpaired Student t test, $P > 0.05$, $n = 6$ /condition).

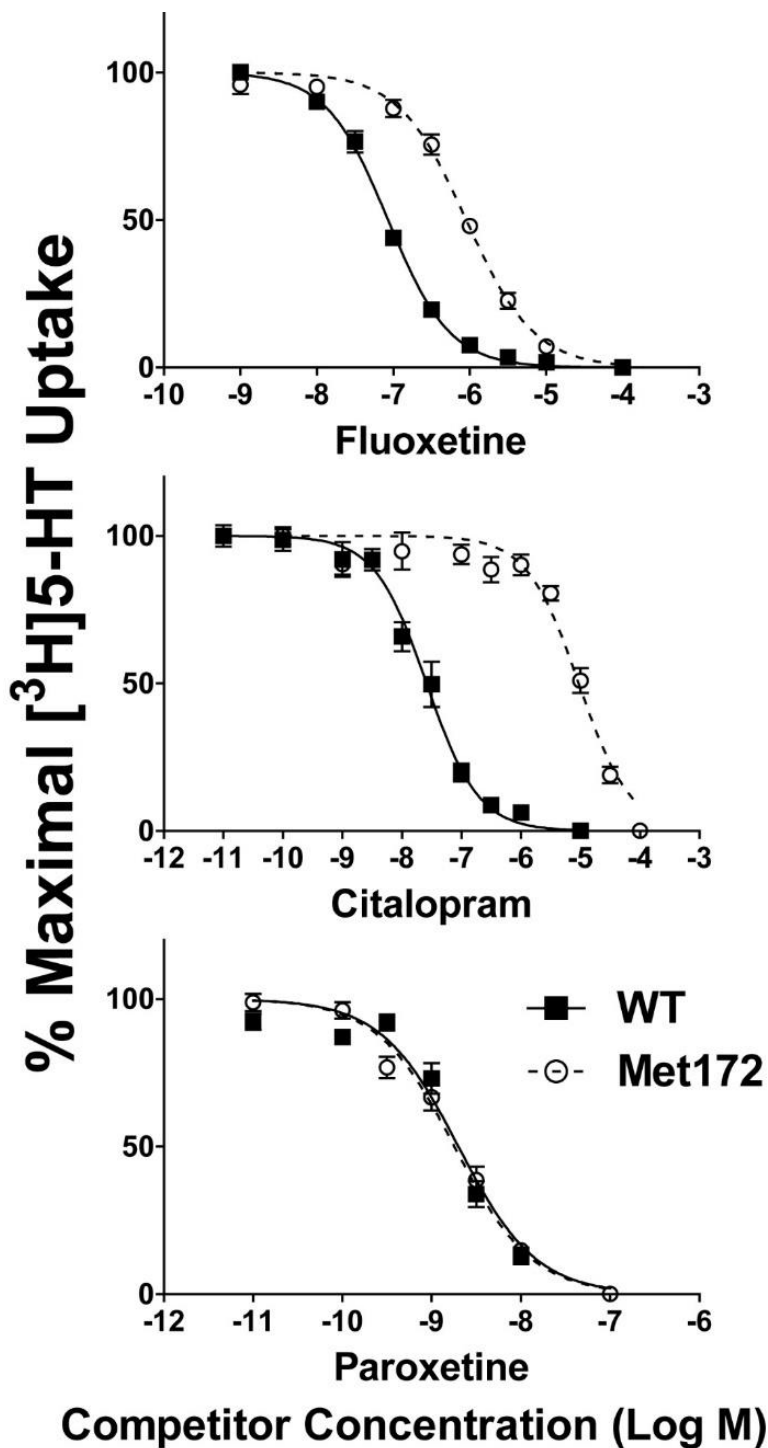


Figure 9. Synaptosomal Competition of 5-HT Uptake. The SERT Met172 mutant imposes altered pharmacological sensitivity with normal SERT expression and function. Competition 5-HT uptake analysis. Fluoxetine (WT $K_I=8.54 \pm 0.02 \times 10^{-8}$ M, SERT Met172 $K_I=9.03 \pm 0.03 \times 10^{-7}$ M), citalopram (WT $K_I=2.07 \pm 0.05 \times 10^{-8}$ M, SERT Met172 $K_I=7.91 \pm 0.05 \times 10^{-6}$ M), and paroxetine (WT $K_I=2.00 \pm 0.03 \times 10^{-9}$ M, SERT Met172 $K_I=1.80 \pm 0.04 \times 10^{-9}$ M) were assessed for their ability to compete with [3 H]5-HT uptake. (n = 4/condition).

In contrast, paroxetine was unaffected by the Met172 variant, as demonstrated by its equivalent potency of 5-HT uptake inhibition in WT, consistent with predictions from transfected cell studies (Henry *et al*, 2006) and prior synaptosomal studies (Thompson *et al*, 2011). These data indicate that the backcrossing onto the C57Bl/6 did not impact the pharmacological sensitivity of the SERT Met172 variant, and additionally that the magnitude of these disrupted sensitivities were preserved from *in vitro* studies as well as the previously assessed 129S6/S4 mouse line containing the SERT Met172 variant. These data indicate that our attempts to dose animals within this ‘dose window’, where a dose of SSRI could fully inhibit WT SERT yet render no functional antagonism on SERT Met172, will be fruitful after these backcrossing efforts.

As an aside, many people have taken interest in the findings that the pharmacological inhibition of paroxetine at SERT is unaffected by the I172M substitution. Of those, most have some training in structural biology, and take these findings as evidence that paroxetine is an allosteric inhibitor of SERT via allosteric binding at the purported S2 SERT binding site (Plenge *et al*, 2012; Plenge and Mellerup, 1985). In our own studies and collaborative efforts, we have observed through computational SERT homology model docking studies that paroxetine does bind at the ‘orthosteric’ 5-HT binding site, where most other SERT blockers are computationally predicted to bind (Kaufmann *et al*, 2009). Moreover, these structures confirm that the stabilized SERT structure bound to paroxetine does not present Ile172 towards the binding pocket in a way that one would predict perturbed paroxetine binding in the presence of the substituted Met172. Based upon these data of the unaltered pharmacological sensitivity in synaptosomes, we can only say that high affinity binding of paroxetine to SERT does not require Ile172. Though our data does not rule out the possibility of S2 binding site activity of paroxetine, together with the homology model docking studies, I do not feel compelled to assert that paroxetine engages the S2 site to

achieve SERT antagonism. Such S2 activity would require perfect and indistinguishable competitive binding for 5-HT, all while remaining completely allosteric of the orthosteric S1 5-HT binding site; I do not feel convinced of that possibility.

Conclusions

The backcrossing the SERT Met172 mouse onto the C57Bl/6 was necessary for further experimentation regarding the acute and chronic SSRI sensitivity studies, though it was necessary to first ensure that the act of backcrossing did not unexpectedly augment any of our foundational assumptions regarding the impacted sensitivity of SERT Met172 to SSRI inhibition with maintained efficacy with respect to 5-HT transport capability. To test this, we chose to validate core facets of these assumptions, namely ensuring that the I172M substitution did not alter the expression of SERT protein, biogenic amine homeostasis, SERT specific 5-HT uptake kinetics, or the predicted altered pharmacology for SSRIs. I was able to validate that these foundational assumptions were not altered following the backcrossing onto the C57Bl/6 genetic background. While we expected these findings, it was necessary to confirm before moving forward onto more complicated queries pertaining to SSRI antidepressant efficacy and the role of SERT antagonism for their actions.

The importance of these findings is multifaceted. Firstly, should we see any differences in SSRI behavioral sensitivity in the SERT Met172 mice, such an observation could be confounded by changes in total SERT protein expression, leading to unbalanced dose administration, and/or altered 5-HT receptor expression or sensitivity. Any minute changes to SERT protein expression basally or over development could dramatically or subtly alter neurodevelopment, which we would

hope to see in gross morphology (data not shown) or monoamine homeostasis, which are unaltered. Such changes could impact basal behavioral effects or sensitivity to SSRIs. Of further importance, the premise of these studies is that we have only altered pharmacology of some SSRIs at SERT without impacting its basal function with respect to 5-HT uptake. Any aberrations in normative function in SERT would create an abnormal state basally, and any conclusions we would draw from SSRI behavioral sensitivity studies could not be attributed solely to deficient SERT binding since many other compensatory effects would also be present in such a mouse. Importantly, I saw no differences in SERT Met172 compared to WT SERT in the 5-HT uptake kinetics, which mirrors what I observed in the biogenic amine level analysis, which would have been altered were SERT kinetics maligned in any functional capacity. And lastly, I was able to show that the backcrossing did not impact the lowered affinities of fluoxetine and citalopram for SERT Met172. These findings indicate that the SERT Met172 mouse, on a biochemical scale, has been validated to a degree to which we can comfortably move forward towards the assessment of SERT specific effects of acute and chronic SSRI sensitivity in a confound-free manner.

CHAPTER III

ACUTE BEHAVIORAL SENSITIVITY OF SERT MET172 TO SSRI

Note: The work presented in this chapter was published as: Nackenoff AG, Moussa-Tooks AB, McMeekin AM, Veenstra-VanderWeele J, Blakely RD (2015). Essential Contributions of Serotonin Transporter Inhibition to the Acute and Chronic Actions of Fluoxetine and Citalopram in the SERT Met172 Mouse. *Neuropsychopharmacology*

Introduction

In the prior chapter, I described studies that show that SERT Met172 protein is expressed as efficiently as WT SERT, can function equivalently to WT SERT with respect to 5-HT uptake, and does not alter brain monoamine homeostasis. Thus, the C57Bl/6 SERT Met172 mouse is validated as a model to assess the functional consequences of the loss of SSRI antagonism of SERT—due to the perturbed affinity for most SSRIs for SERT Met172—in a confound free manner. Provided this shift in pharmacological sensitivity, I should be able to administer a dose of the SSRI that is able to functionally antagonize WT SERT but not SERT Met172 *in vivo*, assuming that the ‘dose window’—the gap between IC₈₀’s of WT and SERT Met172—falls within range of behaviorally relevant doses of SSRIs. Based upon prior validation studies (Thompson *et al*, 2011), we know that a relatively high, behaviorally relevant dose of 20 mg/kg of both fluoxetine and citalopram are able to hit this ‘dose window’, where this dose is able to functionally antagonize WT SERT but not SERT Met172. This would provide the desired situation where the SSRIs fluoxetine and citalopram are present at normal levels in the brain in SERT Met172 mice, yet fail

to engage and functionally antagonize its SERT, leaving those compounds free to engage off-target proteins and receptors that may contribute to antidepressant actions, as some have suggested. In short, since the only differences in SSRI response between WT and SERT Met172 would solely be due to engagement at SERT, we can determine definitively whether SERT antagonism is required for the behavioral efficacy of SSRIs in the acute models of antidepressant efficacy tail suspension test (TST) and forced swim test (FST).

We chose the TST and FST due to their wide adoption and extensive history in the field of antidepressant screening (Crowley *et al*, 2005; Lucki *et al*, 2001; Porsolt *et al*, 1977b; Steru *et al*, 1985). Clinical antidepressants—including fluoxetine, citalopram, and paroxetine—are known to promote escape-like behavior in mice when presented with an inescapable stressor, such as suspension by the tail (TST) or placement in a cylinder of water (FST). These tests evolved from early depression models, notably the original canine learned-helplessness shuttlebox assay (Maier and Seligman, 1976; Overmier and Seligman, 1967). This model could reliably produce a depression-like state in animals that are presented a series of inescapable shocks, to a point where an opportunity to escape is presented, they engage in maladaptive behavior termed ‘learned helplessness’ whereby they fail to seek the escape from the now escapable shock grid. This assay was then adapted for small rodents and found to produce similar results (Seligman and Beagley, 1975). Animals treated with antidepressants are more likely to engage in escape-like behavior than non-treated control animals, in part validating the utility of animal models for human psychotropic drug response (Leshner *et al*, 1979). The disadvantage of the learned helplessness shuttlebox assay is that it requires multiple days of training and repeated sessions to produce the learned helplessness phenotype. In an attempt to produce a quicker developing depression-like state and antidepressant sensitive model, the forced swim test (FST) was developed (Porsolt *et al*, 1977b).

This model involves placing a rodent in a cylindrical container filled partly with water, where the animal swims rapidly in escape-like exploratory behavior until it acquires a ‘learned helplessness’ phenotype and floats, usually over the course of a single 6-minute session. Originally this test was performed in rats, which require 2 total sessions on sequential days to observe an antidepressant induced increases in escape-like swimming behavior (Porsolt *et al*, 1977b). Mice only require one single 6-minute session to observe the antidepressant sensitive escape-like swimming behavior (Porsolt *et al*, 1977a). Animals treated with a single dose of antidepressant will engage in more escape-like swimming behavior, traditionally plotted as a reduction in immobility time due to the predominant immobility/floating behavior in this assay. Additionally, another rapid antidepressant sensitivity screen was developed inspired by Porsolt’s FST, denoted the tail suspension test (TST) (Steru *et al*, 1985), involving the suspension of a mouse by its tail, representing the inescapable stress, and monitoring the mobility and escape-like behavior like in the FST. This assay could not utilize rats as their increased body weight precludes the successful ability to suspend them by their tail for the time required in this assay. Antidepressants suppress the ‘learned helplessness’ phenotype of simply hanging and instead promote rapid movement of escape-like behavior. The antidepressant-like effect in these assays is usually described as ‘resiliency’ due to the promotion of increased escape-like behavior and suppression of the ‘learned helplessness’, as seen through the lens of the original shuttlebox assay. Though one could argue that this escape-like behavior in these assays is maladaptive as floating/hanging conserves energy, especially considering that the water, or suspension by the tail, while unpleasant and represents a potential predator stress environment, is not an equivalent stressor to the shock grid. It is this reason that the FST and TST loses some face and construct validity that the shuttle box assay possesses. But when these assays lose these types of validity, they importantly gain practical utility and retain the predictive validity

of antidepressant sensitivity. This predictive validity is most significant, in that drugs that are clinically effective antidepressants will reliably produce behavioral effects in the FST and TST, which is the major reason why they are still widely utilized in the field of antidepressant research as well as in pharmaceutical antidepressant drug development.

Previous efforts in the lab sought to assess the functional requirement of SERT antagonism for the SSRI antidepressant effect in the TST and FST. Unfortunately, the 129S6/S4 genetic background of the SERT Met172 mice in those studies precluded their usefulness in these acute behavioral SSRI sensitivity assays, either due to poor effect size or directionality of the SSRI induced behavioral effect. In hopes of optimal interpretation of the SERT dependency of SSRI actions, we backcrossed this SERT Met172 mouse onto a more suitable background for behavioral assays. In this effort, we repeat those attempts to ascertain whether acute behavioral sensitivity to SSRIs require SERT antagonism, utilizing the predictively valid TST and FST behavioral assays.

Animal Procedures

All behavioral assays were performed upon behaviorally naïve 8-12 week old male mice in the Neurobehavioral Core lab at the Vanderbilt University Medical Center. Animals were held at 12 hour light:dark cycle (light cycle: 7:00 to 19:00 hours), and all experimentation occurred between 13:00 and 18:00 hours. Animals were transferred to housing within the facility and allowed to acclimate for at least 1 week prior to behavioral manipulations. For acute drug studies, animals were acclimated to handling the day prior to experimentation, at which point body weight was measured for drug dosing considerations. All drugs for acute drug studies were prepared fresh and administered in 0.9% sterile saline solution at 20 mg/kg (10 μ L/g body weight). This dose was

utilized to match our prior studies where we demonstrated significant functional SERT occupancy *in vivo* in WT but not SERT Met172 mice (Thompson *et al*, 2011) as assessed by microdialysis studies, and representing a relatively high behaviorally relevant dose (Crowley *et al*, 2005), which would maximize the potential to detect off-target effects. All experiments and data analyses were scored manually utilizing instantaneous sampling and binning methods of scoring while performed blind to drug condition and genotype.

Tail Suspension Test (TST)

Method

Mice were allowed to acclimate for 1 hour in the testing room at full white room illumination, away from the main colony. Mice were injected (i.p) with saline vehicle, fluoxetine, citalopram, or paroxetine 30 min before a 6 minute TST. The apparatus allows for multiple mice to be run at the same time, though mice are visually isolated from each other in 5 sided opaque plexiglass boxes, with the open side facing towards the room for video capture and handling purposes. Thirty minutes after injection, mice were then suspended by taping the tail to a vertical aluminum bar and activity was recorded by video. Immobility was manually quantified as the time when mice are motionless, excluding minute limb movements. Data was analyzed via two-way ANOVA and Bonferroni post-hoc tests (Graphpad Prism 6.0).

Results

Having validated the C57BL/6J SERT Met172 model as having normal SERT expression and function, yet disrupted *ex vivo* sensitivity to the SSRIs fluoxetine and citalopram, we moved

to assess the efficacy of these drugs in behavioral assays that are known to be sensitive to acute antidepressant administration (Porsolt *et al*, 1977b; Steru *et al*, 1985). WT and SERT Met172 mice display equivalently high rates of immobility in the TST. As expected, WT mice showed a robust decrease in immobility following acute fluoxetine and citalopram administration (**Figure 10**), with effect sizes that match those observed in the literature accounting for age, genetic background strain, and drugs utilized (Crowley *et al*, 2005). SERT Met172 mice, however, displayed no response to either drug (**Figure 10**). Importantly, paroxetine equivalently suppressed immobility in WT and SERT Met172 mice in the TST, consistent with the inability of the Met172 substitution to perturb paroxetine interactions. This important control also demonstrates a normal capacity of the SERT Met172 mice to translate SERT inhibition and elevations in extracellular 5-HT into enhanced mobility in both tests.

Forced Swim Test (FST)

Method

Mice were allowed to acclimate for 1 hour in the testing room at full white room illumination, away from the main colony. Mice were injected (i.p) with saline vehicle, fluoxetine, citalopram, or paroxetine 30 min before a 6 minute FST. Mice were placed in the center of a 15 cm diameter clear plexiglass cylinder filled with tap water (25-27 °C) to a depth of approximately 15 cm for the 6 minute FST and activity was recorded by video.

Tail Suspension Test

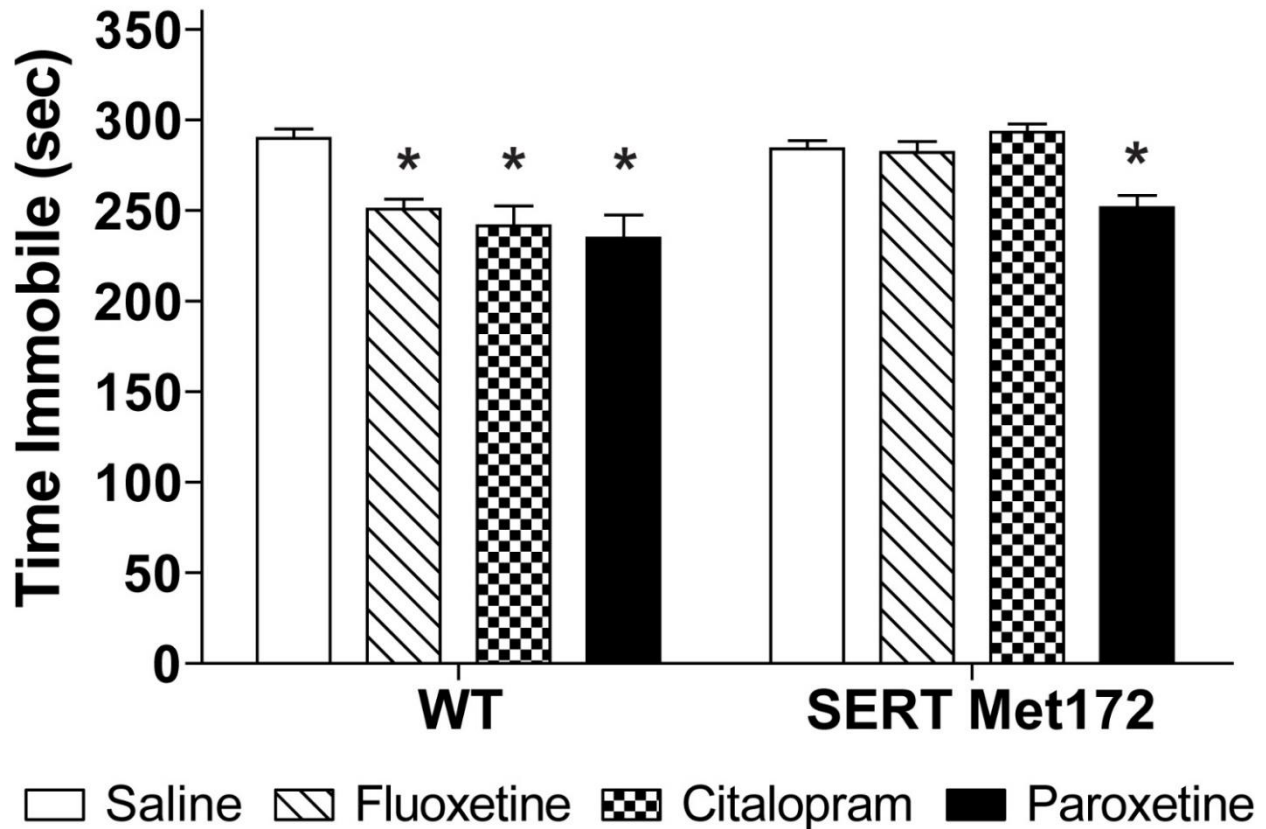


Figure 10. Tail Suspension Test. Actions of fluoxetine and citalopram in the TST arise from SERT antagonism. All tests were performed 30 min after i.p. injection of 20mg/kg drug. Time immobile in a 6-minute TST. Two way ANOVA revealed significant main effects of genotype ($F(1,72) = 17.11, P < 0.05$), treatment ($F(3,72) = 10.82, P < 0.05$), and interaction effect ($F(3,72) = 4.62, P < 0.05$). WT mice display significant decreases in immobility time in response to all drug treatments. Met172 mice display significant decreases in immobility time only in response to paroxetine. * indicates significance ($P < 0.05$) compared to vehicle treatment via Bonferonni posttests ($n = 10-12$ per genotype and condition).

Multiple mice were assessed at the same time, though mice were kept visually unaware of the other mice via black plastic separators. Immobility was manually quantified as the time when mice floated or only made movements to maintain balance. Data was analyzed via two-way ANOVA and Bonferroni post-hoc tests (Graphpad Prism 6.0).

Results

Given that the FST and TST report presumably identical effects of acute SSRI administration, we expected similar findings in the FST as we observed in the TST. In the FST, both WT and SERT Met172 mice display high rates of immobility, given that C57Bl/6 display the highest rates of immobility of the most commonly experimentally utilized mouse strains (Crowley *et al*, 2005; Lucki *et al*, 2001). We found that WT animals display reduced immobility time following administration of all fluoxetine, citalopram, and paroxetine. Like the results in the TST, SERT Met172 mice are behaviorally insensitive to fluoxetine and citalopram (**Figure 11**). Additionally, SERT Met172 mice are still responsive to paroxetine, due to the antidepressant's pharmacological insensitivity to the I172M substitution; this responsiveness to paroxetine also shows that our SERT Met172 animals are behaviorally responsive to traditional monoaminergic antidepressants as well as showing that our behavioral paradigm was effective for the SERT Met172 mice. These results fully mirror what I observed in the TST, namely that SERT antagonism—which is functionally lost in fluoxetine and citalopram in the SERT Met172 model—is required for the acute behavioral effects of SSRIs.

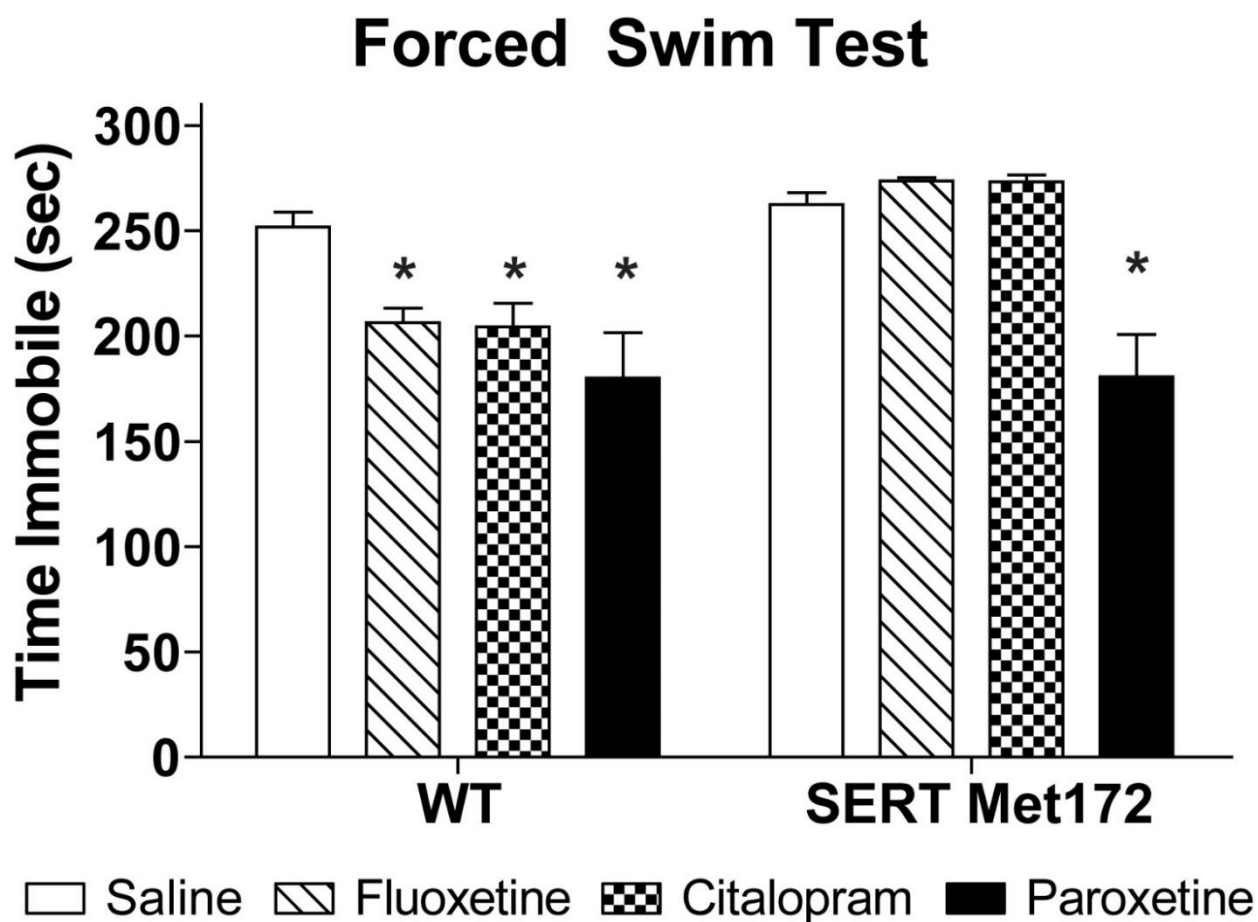


Figure 11. Forced Swim Test. Actions of fluoxetine and citalopram in the FST arise from SERT antagonism. All tests were performed 30 min after i.p. injection of 20mg/kg drug. Time immobile in a 6-min FST. Two way ANOVA revealed significant effects of genotype ($F(1,71) = 18.80, P < 0.05$), treatment ($F(3,71) = 15.92, P < 0.05$), and interaction effect ($F(3,71) = 4.52, P < 0.05$). WT mice display significant decreases in immobility time in response to all drug treatments. Met172 mice display significant decreases in immobility time only in response to paroxetine. For A-B, * indicates significance ($P < .05$) compared to vehicle treatment via Bonferonni posttests ($n = 10-12$ per genotype and condition).

The only differences that we observed between the TST and FST are that the FST has a larger effect size than the TST (Crowley *et al*, 2005; Lucki *et al*, 2001). When analyzing behavioral sensitivity to SSRIs in C57Bl/6 mice, it could be useful to utilize the FST in favor of the TST, due to the C57Bl/6 displaying among the highest rates of immobility in these assays as well as the smallest behavioral response to SSRIs (Crowley *et al*, 2005; Lucki *et al*, 2001). Due to the high rates of use of C57Bl/6 mouse lines because of their extensive utility in various mouse behavioral, genetic, and proteomic analyses, the field should be aware of potential false negative findings of antidepressant properties of drugs if only using the TST for procedural convenience. I have encountered many reports of negative results in the TST with known SSRIs (various personal communications), supporting a strain-dependent limitation of the TST.

Conclusions

With the biochemical validation showing that the I172M substitution does not impact SERT expression or function, the first question we wanted to answer was whether the loss of SERT antagonism of SSRIs, brought about via SERT Met172, would ablate acute behavioral sensitivity in traditional preclinical tests of antidepressant sensitivity. In other words, we wished to ascertain whether SERT antagonism is required for SSRI-mediated antidepressant efficacy. In this aim, I utilized behavioral assays that are only sensitive to a single administration of SSRIs, namely the TST and FST, which are utilized by the pharmaceutical industry for antidepressant drug screening efforts.

The reason behind this motivation is due to a multitude of reports exist describing the interactions of SSRIs with non-SERT targets at physiologically relevant concentrations. When

these targets are manipulated specifically, they have been found to recapitulate antidepressant-like effects in the TST and FST. Furthermore, certain gene knockout studies deleting these non-SERT receptors have provided evidence for a role in the antidepressant effect of SSRIs in TST and FST, suggesting SERT as unnecessary for the acute antidepressant-like effects of SSRIs. Although there have been studies that show that knocking out SERT ablates the behavioral sensitivity of SSRIs in the FST and TST (Holmes *et al*, 2002), the SERT knockout studies are plagued by compensatory alterations that produces a non-native environment in which these SSRIs are being evaluated. In short, there is no definitive reason to assume conclusively from the SERT knockout studies that SERT is a requisite component of SSRI antidepressant action.

Here, we utilize the SERT Met172 model, which is devoid of the confounds that are present in the SERT knockout model. We tested whether SERT is required for acute behavioral efficacy of two SSRIs, citalopram and fluoxetine. We administered these drugs to both WT and SERT Met172 mice using relatively high behaviorally relevant dose of the drugs, including paroxetine as a positive control. The dose we utilized (20 mg/kg i.p.) was chosen due to prior work that showed that this dose fell within the ‘dose window’ and attained separation between WT and SERT Met172 with respect to functional SERT antagonism, and due to our desire to maximize any potential for non-SERT interactions and contributions to behavioral endpoint analysis. In these efforts, I found that all three SSRIs are able to reduce immobility time in both the TST and FST, relative to saline treated control mice, as expected since this is a well characterized feature of antidepressants in these assays. SERT Met172 mice, however, failed to respond to both fluoxetine and citalopram, whereas paroxetine was able to significantly reduce immobility time. Paroxetine is capable of this feat in SERT Met172 mice because of its insensitivity to the I172M substitution, and also serves as a positive control acting as an active SSRI in these SERT Met172 mice, proving

that these knock-in mice are still capable of a traditional serotonin mediated antidepressant-like effect. What these experiments show is that by only changing the pharmacological sensitivity of SERT, thus otherwise retaining a physiologically and neurologically normal mouse, these other non-SERT targets that are theoretically being engaged in both the WT and SERT Met172 mouse are insufficient alone to produce an antidepressant effect. Conversely, we can show definitively with the most specific assay to date that SERT is required for the acute behavioral sensitivity to the SSRIs fluoxetine and citalopram.

CHAPTER IV

BEHAVIORAL AND BIOCHEMICAL SENSITIVITY OF SERT MET172 TO CHRONIC SSRI

Note: The work presented in this chapter was published as: Nackenoff AG, Moussa-Tooks AB, McMeekin AM, Veenstra-VanderWeele J, Blakely RD (2015). Essential Contributions of Serotonin Transporter Inhibition to the Acute and Chronic Actions of Fluoxetine and Citalopram in the SERT Met172 Mouse. *Neuropsychopharmacology*

Introduction

In our prior studies, we were able to demonstrate that the acute behavioral responses to SSRI antidepressants (fluoxetine and citalopram), require SERT antagonism. These findings are important in their own right, but are solely derived from acute single dose responses, meaning that the results we are observing are likely due to the supraphysiological increases in 5-HT signaling brought about via functional SERT antagonism by the SSRIs in the central nervous system (CNS). Whereas SSRIs are able to penetrate the CNS in both mice and human within 1 hour of peripheral drug administration, humans do not experience clinical remediation of depression symptoms after a single administration of SSRI. In fact, SSRI antidepressant pharmacotherapy requires, minimally, 4 weeks of chronic administration before any clinically measurable antidepressant effects are observable.

The temporal disconnect between human antidepressant clinical efficacy (4 weeks) and the behavioral sensitivity in the TST and FST assays is cause to evaluate whether they reflect manipulation of the same biological phenomena. This temporal incongruity suggests that human

clinical efficacy requires much more than just the increases in extracellular 5-HT brought about following a single acute SSRI administration. Although the TST and FST have predictive validity for clinical utility of antidepressant efficacy, these tests do not accurately reflect or produce the biological changes that are required for human clinical efficacy, and thus are not the most valid models for measuring clinically relevant aspects of antidepressant mechanisms. The TST and FST are utilized in the field of antidepressant pharmacology for their ease of use, require no training of the animals, and most importantly because they have predictive validity for agents that are clinically efficacious as antidepressants. Because the TST and FST do not require chronic SSRI administration, they cannot accurately produce or measure the necessary biological components or adaptations that are required for chronic antidepressant efficacy. It is for this reason that we sought tests that are sensitive only to chronic—but not acute—SSRI administration. What could be true for the requirements in acute SSRI sensitivity assays may not hold true for the molecular requirements for chronic SSRI sensitivity. Namely, while SSRIs require SERT antagonism for acute behavioral efficacy in the TST and FST, there is no guarantee that SERT antagonism is required for chronic behavioral sensitivity to SSRIs.

In this aim, we implemented the novelty induced hypophagia (NIH) assay, which is a subtle anxiogenic procedure in which animals reduce food consumption in a novel environment (Dulawa and Hen, 2005). These assays have been utilized for the screening of traditional anxiolytics, but have also been shown to be sensitive to chronic—but not acute—antidepressant administration. The premise of the assay is that the mouse is trained to consume a palatable substance in a stress-free manner in its home cage under low light conditions, which are not stress inducing. On the test day, the mouse is transferred to a new cage that lacks bedding, and the cage is placed on a relatively reflective surface in a room with bright white lights, but in the presence of the same palatable

substance that they have been trained to consume. These sets of conditions produce an anxiety-like state, and promotes anxious exploratory behavior. The major endpoint measure is the latency of the mouse between when it was placed in the novel anxious environment and when it first licks the palatable substance.

The NIH test is a modification of the novelty suppressed feeding (NSF) task, and as such utilizes a liquid palatable substance where the NSF assay uses a small single food pellet (Dulawa and Hen, 2005). Because of this, one can also measure the total amount of palatable liquid consumed over the 30-minute test, which can also tap into the hedonic desire of the animal, a trait that is stimulated following successful antidepressant regimens in human patients. The other advantage of the NIH modification is it requires no food deprivation/restriction of the animal. This reduces the stress put on the animal, as well as providing a more natural environment for which to measure this potential hedonic drive. The NSF assay does not have this luxury since hunger, brought about by food restriction, is a strong motivating force, and thus can reduce the predictive value and effect size of the behavioral assay. Chronic antidepressants, in this assay, promote the reduction in latency to consume the palatable substance as well as increase the total amount consumed in the novel cage. These results indicate the anxiolytic component of the antidepressant effect that is often seen in addition to antidepressant effects in the clinic.

Based upon the necessity for chronic SSRI administration for behavioral efficacy in the NIH test, this assay already shows some face and construct validity than that of either the TST or FST, and therefore much more suitable as a behavioral model of antidepressant efficacy. But it is also important to consider the other factors that are evident upon chronic SSRI administration, which could further illuminate whether the NIH assay is a suitable test to measure chronic antidepressant sensitivity. Following the discoveries of adult neurogenesis in rodents, it was

further discovered that adult neurogenesis can indeed happen in other mammals, including humans, in regions including the hippocampus (Eriksson *et al*, 1998; Malberg *et al*, 2000; van Praag *et al*, 2002). These findings led investigators to determine the neurological utility of adult neurogenesis, and why the brain would keep a stable pool of adult neural progenitor stem cells. Further efforts in the field of depression research found that depressed patients exhibit reduced hippocampal volume, and that antidepressant therapy can rescue this phenotype and recover these volume losses in successfully treated individuals. In animal studies, it was shown that chronic stress paradigms reduced basal rates of hippocampal neurogenesis, and that chronic antidepressant drug administration could recover rates of hippocampal neurogenesis to normal levels (Murray *et al*, 2008; Warner-Schmidt and Duman, 2006). In unstressed animals, chronic SSRI administration—but not acute—could stimulate rates of hippocampal neurogenesis, and on the same timescale of human clinical efficacy (Malberg *et al*, 2000).

Though these findings are intriguing, it was unclear whether adult hippocampal neurogenesis was a coincidental result of chronic antidepressant pharmacotherapy or whether it indeed was a necessary intermediate mechanism of antidepressant efficacy. To test the requirement of hippocampal neurogenesis for the behavioral actions of SSRIs, the Hen group decided to block adult neurogenesis during chronic antidepressant pharmacotherapy in mice and then test whether mice were still behaviorally responsive to chronic SSRI (Santarelli *et al*, 2003). To accomplish this, they chose to focally X-ray irradiate a vertical column (masked by lead shield) of the mouse brain that covered the hippocampus. The X-irradiation induces double stranded DNA breaks, while the largely senescent CNS would be largely unaffected, much like cancer therapy only the highly proliferative cells would be disrupted to a significant degree. And because these neurogenic events require mitosis from the neural stem cell pool before maturation and functional integration into the

hippocampus, the X-irradiation is able to prevent successful mitotic events from occurring, and thus ablating hippocampal neurogenesis, while leaving the rest of the brain arguably intact. It was under these conditions that they ran the NSF assay to see whether the loss of hippocampal neurogenesis could disrupt behavioral sensitivity to chronic SSRI treatment. What they found is that the animals where hippocampal neurogenesis was prevented were insensitive to chronic SSRI treatment and displayed no antidepressant-like effects in the NSF assay, as opposed to untreated antidepressant administered mice. In sum, these seminal studies showed that adult hippocampal neurogenesis is required for the behavioral efficacy of chronic SSRI administration.

The evidence of adult hippocampal neurogenesis occurring in human patients, the fact that the phenomena is stimulated following chronic SSRI administration, and that it is required for behavioral efficacy in the NSF/NIH test suggests increased face and construct validity of the NIH test for the evaluation of SSRI sensitivity over the acute TST and FST assays. Additionally, this provides a non-behavioral endpoint for analysis for SSRI action and the dependency of SERT for antidepressant actions of SSRIs. While the TST and FST are important quick measures of antidepressant efficacy, we now have at our disposal more construct valid endpoints for which to measure antidepressant efficacy that are more congruent with human clinical features.

Materials

Drugs

For chronic administration studies, drugs were dissolved in tap water and filtered for sterility (Stericup-HV, 0.45 μ m, PVDF; SCHVU05RE, Millipore, Billerica, MA, USA). Drinking

rates were monitored and found to be unaffected between genotypes or by drug treatments (data not shown).

Novelty Induced Hypophagia (NIH)

Method

Animals were trained to consume a palatable substance (Vanilla Ensure®) in their home cage under low red light conditions (~50 lumens) in the testing room for a total of 3 days of 30 min sessions. On the first day of testing, mice were moved to a novel cage with no bedding and high white light illumination (~1200 lumens), where the latency to first consume Vanilla Ensure®, as well as the amount consumed after 30 min (in grams), were measured. On the following day, latency and consumption values of Vanilla Ensure® were assessed in the home cage under low light. To avoid ordering effects, the two testing days were switched for half of the mice. We administered citalopram, fluoxetine, or paroxetine in the drinking water (160 mg/L) for 28 days prior to behavioral screening, which was designed to accomplish a ~20 mg/kg-day chronic dosing regimen (David *et al*, 2009; Santarelli *et al*, 2003; Warner-Schmidt *et al*, 2011). Drug dosage was chosen for continuity with the acute drug sensitivity studies described above, as well as producing drug serum concentrations that are on the high end of clinically relevant serum concentrations (Dulawa and Hen, 2005), maximizing the potential to detect off-target activity. Animals were singly housed during training and testing phases. Data was analyzed via two-way ANOVA and Bonferroni post-hoc tests (Graphpad Prism 6.0).

Results

Although the FST and TST are tests with predictive validity for antidepressant efficacy, we sought to evaluate the SERT-dependency of SERT actions in the Met172 model using tests that more closely mirror the time course associated with SSRI clinical efficacy. Thus, we implemented the NIH test, a behavioral paradigm sensitive to chronic, but not acute, antidepressant administration (Dulawa and Hen, 2005). The NIH test involves monitoring the latency of animals to approach and consume a known palatable substance in a novel, stressful environment, where SSRIs reduce latency and enhance consumption after chronic but not acute administration (Dulawa and Hen, 2005). We administered citalopram, fluoxetine, or paroxetine in the drinking water (160mg/L) for 28 days prior to behavioral screening. Compared to home cage testing, vehicle treated mice display significant increases in the latency to initially consume the palatable substance, upon presentation. Following chronic SSRI administration, WT mice displayed a significantly reduced latency to consume Vanilla Ensure® in the novel cage compared to vehicle condition (**Figure 12**). SERT Met172 mice administered paroxetine displayed similar reductions in latency as WT animals, whereas those provided fluoxetine or citalopram showed no significant reductions relative to vehicle condition. Paralleling our findings with latency, WT mice also displayed increased novel cage consumption following all SSRI treatments whereas SERT Met172 mice displayed increased consumption only with paroxetine administration (**Figure 12**).

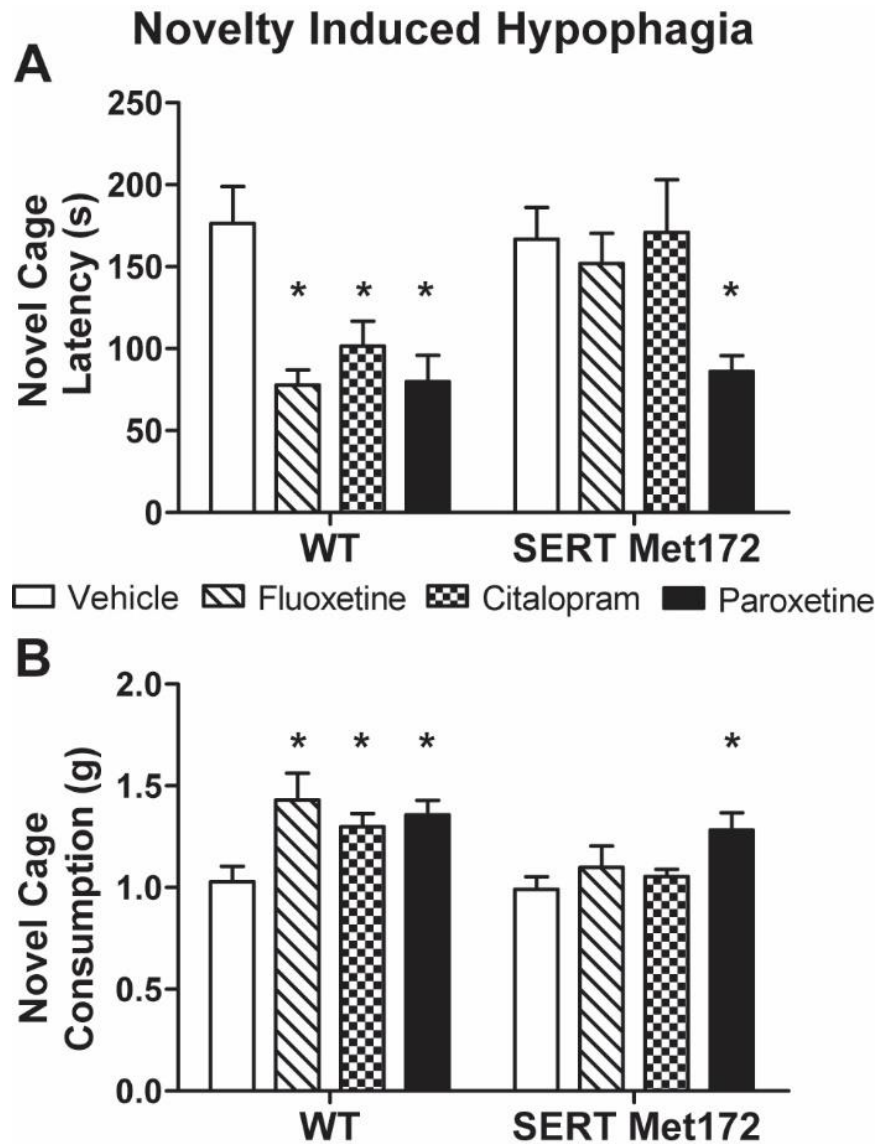


Figure 12. Novelty Induced Hypophagia Test. SERT antagonism is required for the chronic effects of fluoxetine and citalopram in the NIH test. **A:** Latency to consume Ensure® in novel cage was recorded. Two way ANOVA revealed significant main effects of genotype ($F(1,97) = 4.81, P < 0.05$) and treatment ($F(3,97) = 5.97, P < 0.05$), but not a interaction effect ($F(3,97) = 1.92, P > 0.05$). WT mice display significant reductions in latency following chronic SSRI administration, whereas Met172 mice display significant reductions only following paroxetine administration. **B:** Consumption effects: mice were left in novel cage for a total of 30 minutes and allowed to freely consume vanilla Ensure®. Two way ANOVA revealed significant main effects of genotype ($F(1,97) = 8.53, P < 0.05$) and treatment ($F(3,97) = 6.10, P < 0.05$), but not an interaction effect ($F(3,97) = 1.46, P > 0.05$). WT mice significantly increase consumption following chronic SSRI administration. SERT Met172 mice only increase consumption following paroxetine administration. For A-B, * indicates significance ($P < .05$) compared to vehicle treatment Bonferroni post-hoc tests ($n = 12-18$ per genotype and condition)

Hippocampal Neurogenesis

Method

Proliferation: Following chronic administration of SSRIs and behavioral screening in the NIH test, mice were assayed for levels of hippocampal stem cell proliferation. Mice were administered 5-bromo-2'-deoxyuridine (BrdU; 4 x 75mg/kg i.p., every 2hr; Sigma-Aldrich, St. Louis, MO, USA) to pulse label newly proliferating S-phase mitotic cells. 24 hours following the last injection of BrdU, mice were anesthetized via injection of 100 mg/kg i.p. pentobarbital and transcardially perfused with ice-cold PBS, followed by ice-cold 4% paraformaldehyde. Brains were sectioned (40 μ m) via a freezing stage sliding microtome (Leica, SM2000R, Buffalo Grove, IL, USA). Every sixth section of the hippocampus (plates 41-61 (Paxinos and Franklin, 2004)) or the subventricular zone (SVZ; plates 27-40) were immunostained for BrdU incorporation (mouse anti-BrdU; 1:1000; BD#347580; BD Biosciences; Franklin Lakes, NJ, USA) and detected following secondary antibody incubation (biotinylated donkey anti-mouse; 1:500; PA1-28627; ThermoFisher), ABC amplification (VectaStain; Vector Labs; Burlingame, CA, USA), and diaminobenzidine (DAB) detection. Brightfield stitched images were captured (Zeiss Axio Imager.M2) and stored for analysis. BrdU⁺ cells in the subgranular zone (SGZ) of the hippocampus or SVZ were counted using the ITCN (Image-based Tool for Counting Nuclei) plugin for ImageJ (Byun *et al*, 2006) by an observer blinded to genotype and drug treatment. Total counts were extrapolated to whole region analyzed, accounting for the harmonic mean of sections per region and initial sampling limits.

Survival: We injected a separate cohort with BrdU prior to administration of SSRIs. Proliferating cells at steady state were pulse labeled with BrdU (4 x 75mg/kg i.p., every 2hr), and

then their drinking water was then supplanted with SSRI infused drinking water (at 160 mg/L). After 4 weeks of SSRI administration, mice were sacrificed and brain tissue collected and developed using the immunohistochemistry procedures described above. BrdU+ cell quantification for proliferation measures included the SGZ and the granule cell layer of the dentate. Cell counting was performed as described above. Data was analyzed via two-way ANOVA and Bonferroni post-hoc tests (Graphpad Prism 6.0).

Results

Hippocampal neurogenesis is a non-behavioral measure of antidepressant efficacy that has been shown to be sensitive to chronic, but not acute, antidepressant administration, and irradiation studies demonstrate neurogenesis as essential for SSRI efficacy in the NIH test (Santarelli *et al*, 2003). SSRIs promote both hippocampal stem cell proliferation rate and survival of newly generated hippocampal stem cells (Wang *et al*, 2008). Consistent with these studies, chronic administration of fluoxetine, citalopram, and paroxetine robustly stimulated versus vehicle treated mice (~2x increase over vehicle) the proliferation rate (**Figure 13**) of hippocampal stem cells in WT mice, assessed via BrdU+ immunohistochemistry. In contrast, only paroxetine was able to enhance hippocampal stem cell proliferation (**Figure 13B**) in SERT Met172 mice, with similar magnitude of stimulation as seen in WT mice. Importantly, we saw no effect of chronic SSRI administration upon the stimulation of stem cell proliferation in the SVZ across both genotypes (**Figure 14**), concurrent with other studies showing the exclusivity of SSRI stem cell effects to the SGZ (Malberg *et al*, 2000; Santarelli *et al*, 2003).

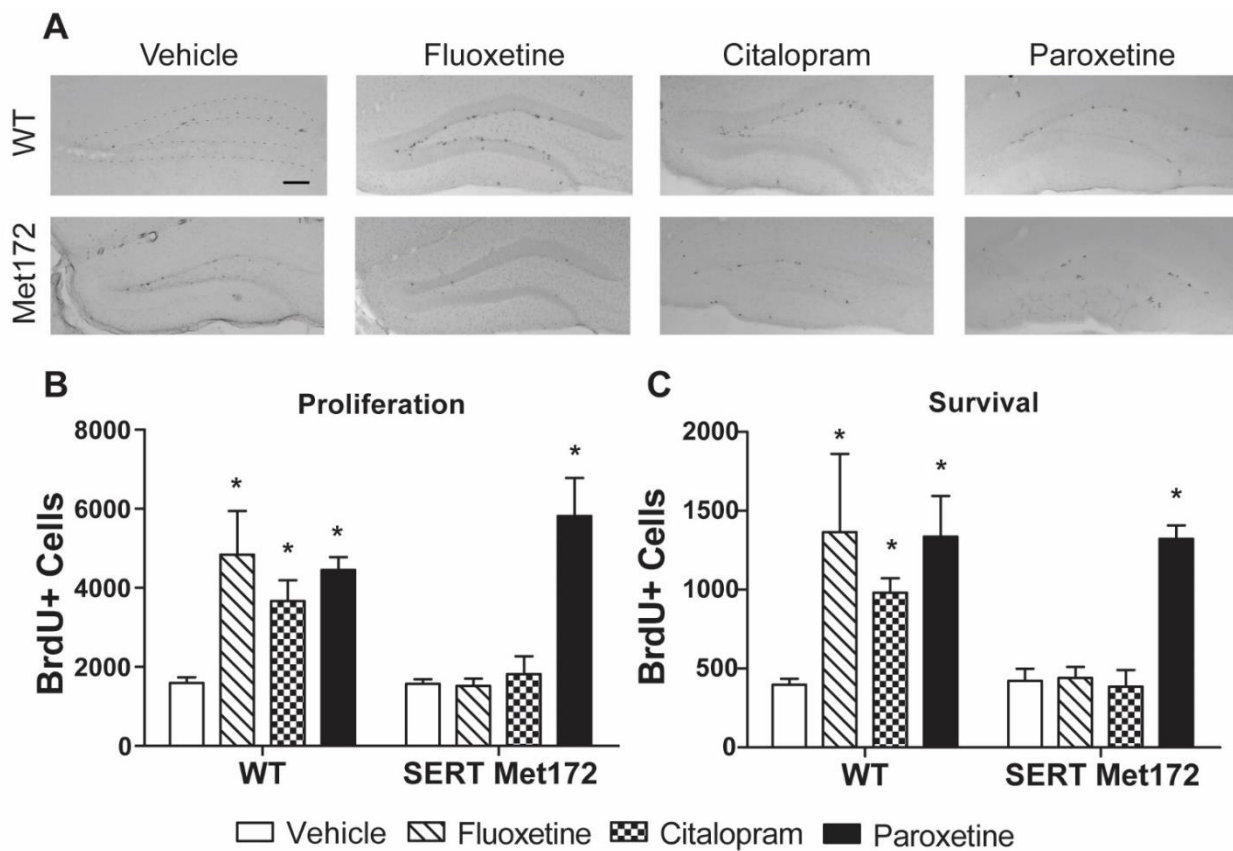


Figure 13. Hippocampal Neurogenesis. SERT antagonism is required for the stimulation of hippocampal stem cell proliferation and survival following chronic fluoxetine and citalopram. **A.** Example images of hippocampal stem cell proliferation studies. Scale bar represents 100 μm . Dotted line represents the border of the granular cell layer of the dentate gyrus for visual aid. **B.** Proliferation: Following administration of SSRIs, WT mice display significant increases in stem cell proliferation rate. Met172 mice display significant increases in proliferation only after paroxetine. Two way ANOVA revealed significant effects of genotype ($F(1,19) = 4.60, P < 0.05$), treatment ($F(3,19) = 8.88, P < 0.05$), and interaction effect ($F(3,19) = 5.06, P < 0.05$). **C.** Survival: Newly generated stem cells survive significantly more in SSRI treated mice than vehicle. SERT Met172 mice display increased survival rates only after paroxetine. Two way ANOVA revealed significant main effect of genotype ($F(1,22) = 6.04, P < 0.05$) and treatment ($F(3,22) = 6.03, P < 0.05$), but not on interaction effect ($F(3,22) = 2.47, P > 0.05$). **For A-C,** * indicates significance compared to vehicle ($P < .05$) following Bonferroni post-hoc tests ($n = 4-5$ per genotype and condition).

SVZ Proliferation

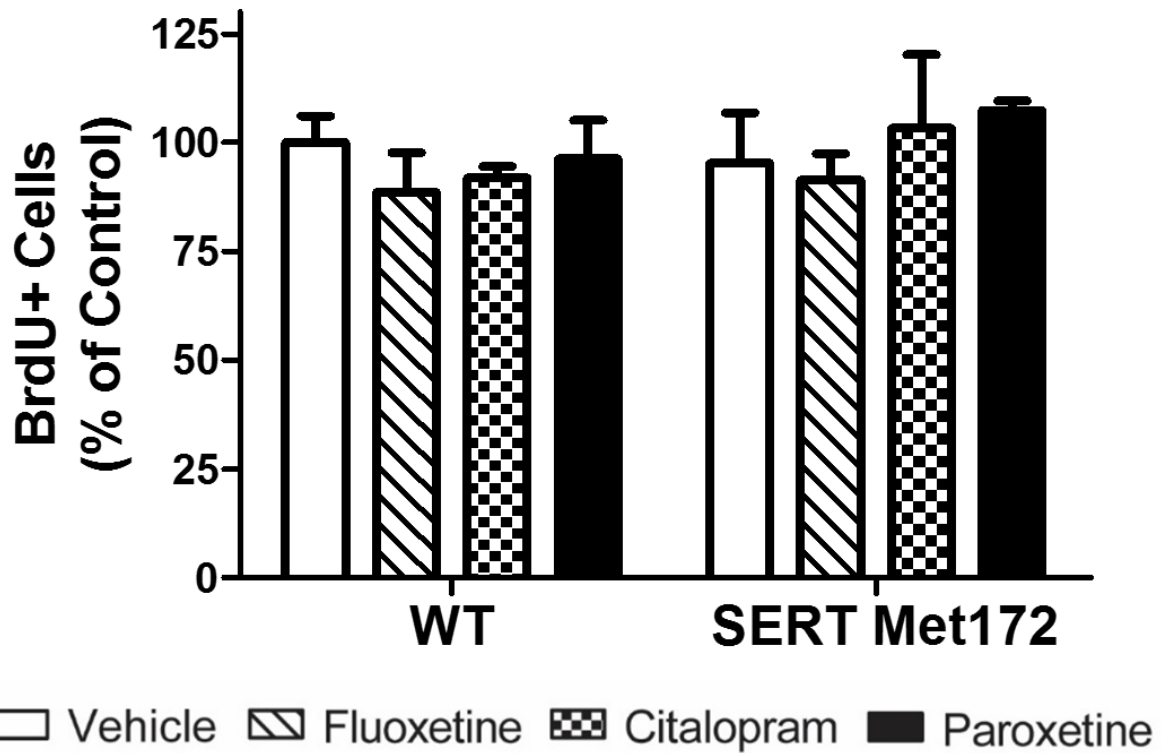


Figure 14. Stem cell proliferation in SVZ. Following chronic SSRI administration, SVZ stem cell proliferation rate (rate analysis 24 hours following BrdU injection) is unchanged, irrespective of genotype. Results are normalized to WT vehicle treated condition. Two-way ANOVA found no significant effects of treatment ($F(1,3) = 0.59, P > 0.05$) or genotype ($F(1,1) = 0.64, P > 0.05$) ($n = 4$ per genotype and condition).

Most basally proliferated stem cells do not survive and functionally integrate into the dentate gyrus of the hippocampus (Eriksson *et al*, 1998; Malberg *et al*, 2000). As such, we observed a roughly 75% pruning of basally proliferated stem cells in vehicle treated mice in our paradigm. WT mice, when chronically administered fluoxetine, citalopram, and paroxetine following BrdU pulse labeling of hippocampal stem cells, display increased survival rates of basally proliferated stem cells (**Figure 13C**). Parallel to the proliferation experiments, SERT Met172 mice do not display increased survival of the basally proliferated hippocampal stem cell pool following administration of fluoxetine and citalopram (**Figure 13C**). SERT Met172 mice, however, do display increased survival of the stem cell pool following chronic administration of paroxetine (**Figure 13C**).

Conclusions

In the previous chapter, I presented the findings concerning the SERT dependence of behavioral effects following acute administration of SSRIs. These analyses were performed as the majority of the assertions of non-SERT targets as responsible for antidepressant efficacy of SSRIs derive from utilizing acute antidepressant sensitive behavioral models. If these non-SERT binding events were important for the behavioral effects of SSRIs in the FST and TST, SSRIs should still be effective in the SERT Met172 mouse model following a single administration of SSRI. SERT Met172 mice, however, do not respond behaviorally to SSRI treatment in the FST and TST, indicating that SERT antagonism is required for the acute behavioral efficacy of SSRIs, and additionally that these non-SERT engagements of SSRIs—should they be occurring—are insufficient alone to produce an antidepressant effect in these acute behavioral models. These

models, as stated previously, are not truly representative and efficacious in testing the chronic nature of SSRI administration that is required for clinical antidepressant efficacy. When SSRIs must be administered at high levels for weeks, this suggests that the simple proposed pharmacological activity at SERT may not be a critical mechanism on a chronic administration timescale, and that other lower affinity targets engaged over the long term may be responsible for the antidepressant effects of SSRIs. This scenario is intriguing considering that SSRIs have been shown to modulate nerve branching and growth in a non-SERT manner (Bonnin *et al*, 2012), and that new nerve growth is important and required for chronic SSRI efficacy (Santarelli *et al*, 2003). Given the shortcomings of the TST and FST for testing these more complicated scenarios, we next moved to test the importance of SERT antagonism onto behavioral and biochemical models that are sensitive to chronic—but not acute—SSRI administration.

Here, I first test the ability of SSRIs to induce antidepressant-like effects in the NIH test, a model mixing aspects of anxiety and anhedonia, which is sensitive to chronic SSRIs (Dulawa and Hen, 2005; Santarelli *et al*, 2003). On its own, the delayed timescale to antidepressant efficacy is important for establishing some semblance that the NIH test is sensitive to similar biological processes as are occurring in clinical populations taking SSRIs. The NIH test is believed to measure certain core aspects of depression, namely anxiety and anhedonia, both of which are presented in patients afflicted with depression, and are remedied following successful antidepressant treatment. In fact, mice that have been chronically administered SSRIs display reduced anxiety and anhedonia (here hypophagia to a known palatable substance) in the NIH test compared to vehicle treated control mice (Dulawa and Hen, 2005; Santarelli *et al*, 2003), though it is unknown whether this effect of SSRIs requires SERT antagonism. Utilizing the SERT Met172 mouse, we can assess this question due to its selective removal of the ability of SERT blockers to

bind and inhibit SERT, yet retaining all other non-SERT CNS targets for these SSRIs to engage and potentially create antidepressant effects. When I administer SERT Met172 mice SSRIs chronically, these mice do not display any anxiolytic or hedonic-like effects in the NIH. These SERT Met172 mice, in other words, are behaviorally insensitive to chronic SSRI in the NIH test, indicating that, like the acute tests, SERT antagonism is required for the chronic behavioral effects of SSRIs.

Next, we needed to investigate another important facet of chronic SSRI actions, namely their ability to stimulate hippocampal neurogenesis. Hippocampal neurogenesis is ongoing, but decreases with age (Kuhn *et al*, 1996). This process is supported by the proliferation of stem cells in the subgranular zone of the dentate gyrus of the hippocampus, and these cells differentiate into functional neurons and integrate into the dentate gyrus of the hippocampus (van Praag *et al*, 2002), though most do not survive this process (Eriksson *et al*, 1998). Chronic SSRI administration, however, robustly increases the rate of stem cell proliferation and additionally increases the rate of survival among basally proliferated stem cells (Wang *et al*, 2008). The timescale of this enhanced proliferation effect of SSRIs occurs along the timeframe like that required for clinical efficacy of SSRIs, as well as when behavioral effects are seen in the NIH test (Santarelli *et al*, 2003). Additionally, when neurogenesis is prevented, mice are not behaviorally sensitive to SSRIs in the NIH test (Santarelli *et al*, 2003). We thus felt it was an important endpoint to measure in addition to behavioral sensitivity in the NIH test, as some studies have shown neurogenesis independent behaviors that are still sensitive to chronic SSRIs (David *et al*, 2009), as well as strain differences in the requirement of neurogenesis for NIH behavioral efficacy of SSRIs (Holick *et al*, 2007). However, in these studies, we did observe congruent effects of behavioral efficacy and hippocampal neurogenesis in our mice. WT mice, when chronically administered SSRIs display

an increased proliferation rate of hippocampal stem cells. These effects were limited to the SGZ and did not extend to another important stem cell niche of the SVZ (Alvarez-Buylla and García-Verdugo, 2002). These findings corroborate earlier work stating the selectivity of SSRI-induced stem cell proliferation to the SGZ and not the SVZ (Malberg *et al*, 2000; Santarelli *et al*, 2003), further highlighting the importance of the SGZ in the antidepressant response. Basally proliferated stem cells in WT mice are also sensitive to administration of SSRIs, displaying increased survival rates. When I chronically administered fluoxetine or citalopram to SERT Met172 mice, which are behaviorally insensitive in NIH test, these mice display no increase in the proliferation rate or survival of proliferated cells compared to vehicle treated control mice, though SERT Met172 mice are still responsive to paroxetine in both measures.

Taken together, these data from the behavioral NIH test and the effects upon hippocampal neurogenesis indicate that SERT antagonism is required for the chronic behavioral and biochemical effects of SSRIs. While the full pathway and effects brought about by chronic antidepressant administration have not been entirely defined or evaluated, these endpoints have been well characterized by the field to be critical for chronic SSRI efficacy. While these results may not be the most surprising to some, given the well characterized serotonin hypothesis of depression and SSRI antidepressant efficacy, there have been many characterizations of non-SERT SSRI engagements that may be contributing towards antidepressant efficacy, and no other models or assays have been able to dispel these notions or indicate without doubt or caveat that SERT is required for the acute and chronic behavioral and biochemical effects of SSRIs. Our tests, utilizing the SERT Met172 mice, clearly show that SERT is required for the antidepressant effects of SSRIs. These findings represent the most specific analyses to date supporting a role for SERT in both acute and chronic SSRI behavioral and biochemical actions *in vivo*.

CHAPTER V

ANTIDEPRESSANT SENSITIVITY TO THE NOVEL ANTIDEPRESSANT VORTIOXETINE IN SERT MET172

Note: This work was accomplished with the assistance of members of the Blakely laboratory and Lundbeck Research USA, under the direction of Dr. Randy Blakely and Dr. Connie Sanchez, respectively. Chronoamperometry experiments were performed by Dr. Nicole Baganz and microdialysis experiments were performed by Dr. Linda Simmler, within the Blakely laboratory. Slice competition binding experiments were performed by Dr. Alan Pehrson of Lundbeck Research USA.

Introduction

Depression is one of the most common psychological disorders, with incidence rates approaching 7% (Kessler *et al*, 2005), lifetime incidence 17% (Kessler, 2005), and among the leading contributors to global disease burden (Ferrari *et al*, 2013). The most common pharmaceutical treatment options rely upon serotonin selective reuptake inhibitors (SSRI), which act to prevent the serotonin transporter (SERT) from removing serotonin (5-HT) from the synaptic space, thereby prolonging 5-HT signal duration. Despite this, SSRIs have limited utility due to high rates of pharmacological insensitivity (Warden *et al*, 2007) and delayed clinical efficacy. To improve efficacy, a number of drugs have been developed that offer additional target engagements, including other transporters and receptors, though always retaining actions at SERT. This shift represents a desire to develop newer pharmacological treatments based upon updated scientific understanding of the complexities of depression and limitations of existing treatments. One of these drugs is vortioxetine, the most recently approved FDA-approved antidepressant that targets

SERT as well as 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT₃ and 5-HT₇ receptors (Mørk *et al*, 2012). The introduction of these new mechanisms raises the question as to whether the 5-HT receptor actions alone—without SERT inhibition—could be efficacious as an antidepressant. There is precedence for this expectation, given that many 5-HTR directed ligands possess antidepressant-like effects in preclinical animal behavioral studies (Artigas, 2013; Carr and Lucki, 2010). Of note, antidepressant-like effects can be produced by activation of 5-HT_{1A} (Singh and Lucki, 1993), 5-HT_{1B} (Tatarczyńska *et al*, 2005), 5-HT_{2C} (Cryan and Lucki, 2000), and 5-HT₄ (Lucas *et al*, 2010) receptors. Additionally, antidepressant-like effects can be generated via antagonism of 5-HT_{2A} (Patel *et al*, 2004), 5-HT₃ (Ramamoorthy *et al*, 2008), and 5-HT₇ (Wesołowska *et al*, 2006) receptors. Although these 5-HT receptor directed ligands have been shown to be efficacious in preclinical animal studies, none have been proven clinically superior to SSRI antidepressants, either due to poor metabolic profile, limited efficacy, or side effect profile. The study of vortioxetine provides a unique opportunity to question whether it is possible to recapitulate an antidepressant effect without SERT inhibition in a clinically relevant molecule.

In order to selectively remove the SERT component of vortioxetine action, one could use the SERT knockout mouse. This approach would be counterproductive considering the SERT knockout mouse displays reductions in CNS tissue 5-HT levels (Bengel *et al*, 1998), 5-HT neurons (Lira *et al*, 2003), and altered dorsal raphe firing rates (Lira *et al*, 2003). Particularly concerning are the observed changes in 5-HT receptor expression in SERT knockout mice (Cour *et al*, 2001; Fabre *et al*, 2000; Li *et al*, 2000; Rioux *et al*, 1999). Were we to utilize the SERT knockout mouse, our findings of potential SERT-independent efficacy would be confounded, for example, by the alterations in 5-HT_{1A} expression—one of the major non-SERT targets of vortioxetine—and would be clinically non-generalizable.

Our approach to ameliorate this problem is to utilize the SERT Met172 model, a novel transgenic mouse model that disrupts high-affinity binding of SERT antagonists without affecting 5-HT transport capability at SERT, preserving monoamine homeostasis (Nackenoff *et al*, 2015; Thompson *et al*, 2011). Through this approach, we will be able to remove the actions of vortioxetine at SERT but preserve its effects at other 5-HT receptors in a system devoid of developmental alterations (**Figure 15**). Should vortioxetine remain behaviorally efficacious in SERT Met172 mice, this would indicate that SERT may not be a critical target for future development of 5-HT directed antidepressants, and that these future efforts would have more freedom pursuing specific 5-HT receptors to recapitulate the traditional antidepressant response, potentially presenting lesser side-effect profile and quicker clinical efficacy.

Animals

SERT Met172 knock-in mice were created as described previously (Thompson *et al*, 2011). These animals were then backcrossed onto a C57BL/6 genetic background for future utility in behavioral assays and described previously (Nackenoff *et al*, 2015). Due to functional coding differences between 129S6 and C57BL/6 mice in the 5-HTT gene (Carneiro *et al*, 2009), the ‘WT’ animals here are the result of parallel backcrossing of 129S6 mice onto the C57BL/6 genetic background, preserving the 129S6 5-HTT polymorphisms (Nackenoff *et al*, 2015). Animals used in all experiments were male mice aged 8-12 weeks (at time of beginning of experimental manipulation) generated from homozygous WT and knock-in (SERT Met172) breeders. All animal studies were performed in accordance with protocols approved by the Vanderbilt University animal care and use committee.

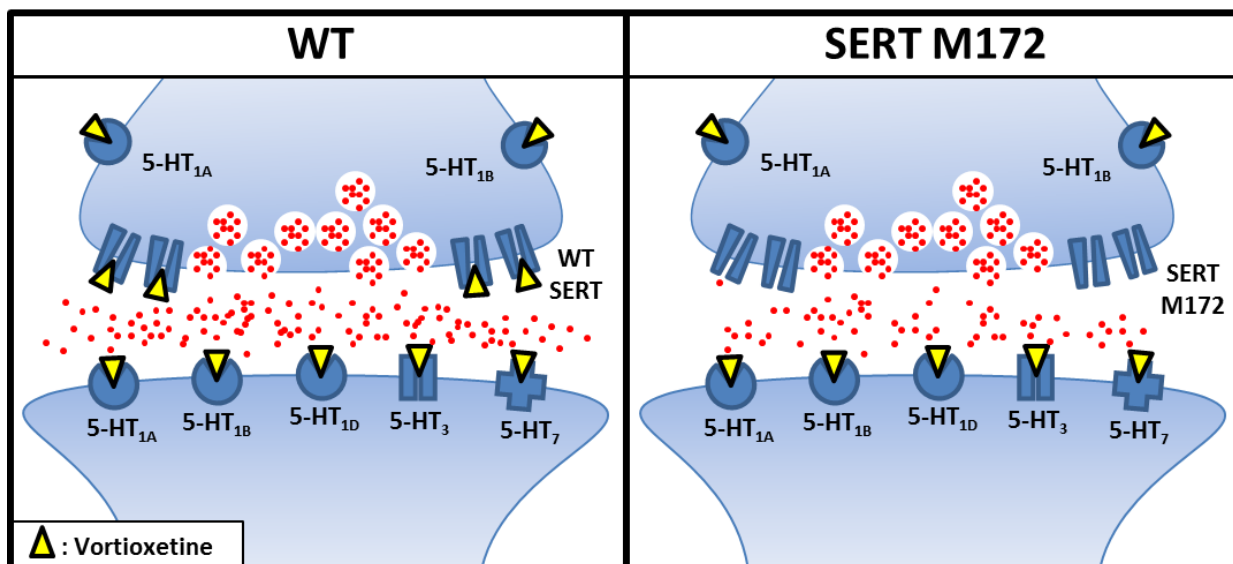


Figure 15. Research Strategy for Vortioxetine in SERT Met172. Cartoon diagram of combined effects of vortioxetine in WT and SERT Met172. Vortioxetine is sensitive to the SERT Met172 substitution. Here, we propose the potential maintained efficacy of vortioxetine in the SERT Met172 model due to its maintained actions directly at 5-HT receptors

Drugs

Vortioxetine HBr was provided by Lundbeck Research USA (Paramus, NJ, USA), and Paroxetine HCl was procured from TCI Chemicals (Portland, OR, USA). For *in vitro* and *ex vivo* studies, vortioxetine was prepared at 10mM stock solutions in 100% DMSO. Final DMSO concentrations for these studies were under 1%. All control conditions contained the highest level of DMSO that would be presented by the most concentrated vortioxetine solution. For acute animal studies, vortioxetine was prepared fresh at 5 and 10mg/kg (free base) and paroxetine at 20 mg/kg, dissolved in 10% β -cyclodextrin/0.9% sterile saline solution (vehicle), sterile filtered (0.2 μ m syringe filter, 195-2520, ThermoScientific, Waltham, MA, USA), and administered intraperitoneal (i.p.) at 10uL/g animal weight.

In Vitro Analysis of Vortioxetine Competition of 5-HT Uptake

Method

HEK-293T cells were cultured at 37°C in modified DMEM cell media containing 10% dialyzed fetal bovine serum (to eliminate 5-HT in growth media) and antibiotics. Cells were transfected with 5 μ g of plasmid DNA from either WT hSERT or SERT Met172 containing pcDNA3 plasmids (Invitrogen). Plasmid DNA was preincubated and loaded into artificial membrane using TransIT (3 μ L per μ g DNA; Mirus, Madison, WI, USA) for 15 minutes. A cellular suspension containing 5 x 10⁶ cells in cell growth media was then added to this transfection mixture and incubated for 30 minutes, allowing the artificial membranes to fuse and deliver plasmid DNA into the cells. Afterwards, the cell and transfection suspension was loaded onto poly-D-lysine

coated (to aid adherence) 12 well plates. A separate equal aliquot was plated in a separate plate for protein determination at the time of uptake, affording normalization by cellular protein levels. The cells were allowed to plate, grow, and express SERT from the plasmid DNA for 48 hours. At the time of the radiouptake assay, cell media was replaced with Krebs-Ringer HEPES buffer (KRH) assay buffer containing pargyline (MAOI, prevents enzymatic metabolism of 5-HT), and ascorbic acid (preservative; prevents spontaneous oxidation of 5-HT). Cells were then preincubated with various concentrations of vortioxetine for 10 minutes at 37°C. Cells were then incubated with 20 nM [³H]5-HT (PerkinElmer, NET498001MC, Waltham, MA, USA) and allowed to accumulate 5-HT for 10 minutes at 37°C. Assay buffer was then aspirated off and cells were washed three times in assay buffer. Cells were then solubilized in scintillation fluid (Microscint 20, 6013621, PerkinElmer) overnight, before scintillation spectroscopy (Topcount, PerkinElmer). Uptake counts were normalized to protein concentration (Pierce BCA; ThermoFisher), averaged across experiments, and limited to maximum (uninhibited 5-HT uptake condition) and minimum via full block (1 μM paroxetine; defines non-specific uptake) conditions. Competition curve was fit to a single site inhibition model (Graphpad Prism 6.0).

Results

The SERT I172M substitution disrupts affinity for many SERT directed ligands without affecting 5-HT affinity or uptake via SERT (Henry *et al*, 2006; Thompson *et al*, 2011). We were able to determine that vortioxetine is indeed sensitive to the SERT I172M substitution (**Figure 16**), and that the shift in inhibition efficacy (~20 fold) is on par with that of fluoxetine, which we have demonstrated that can achieve functional SERT inhibition at a single dose in WT but not in SERT Met172 mice (Nackenoff *et al*, 2015; Thompson *et al*, 2011).

In Vitro Competition 5-HT Uptake

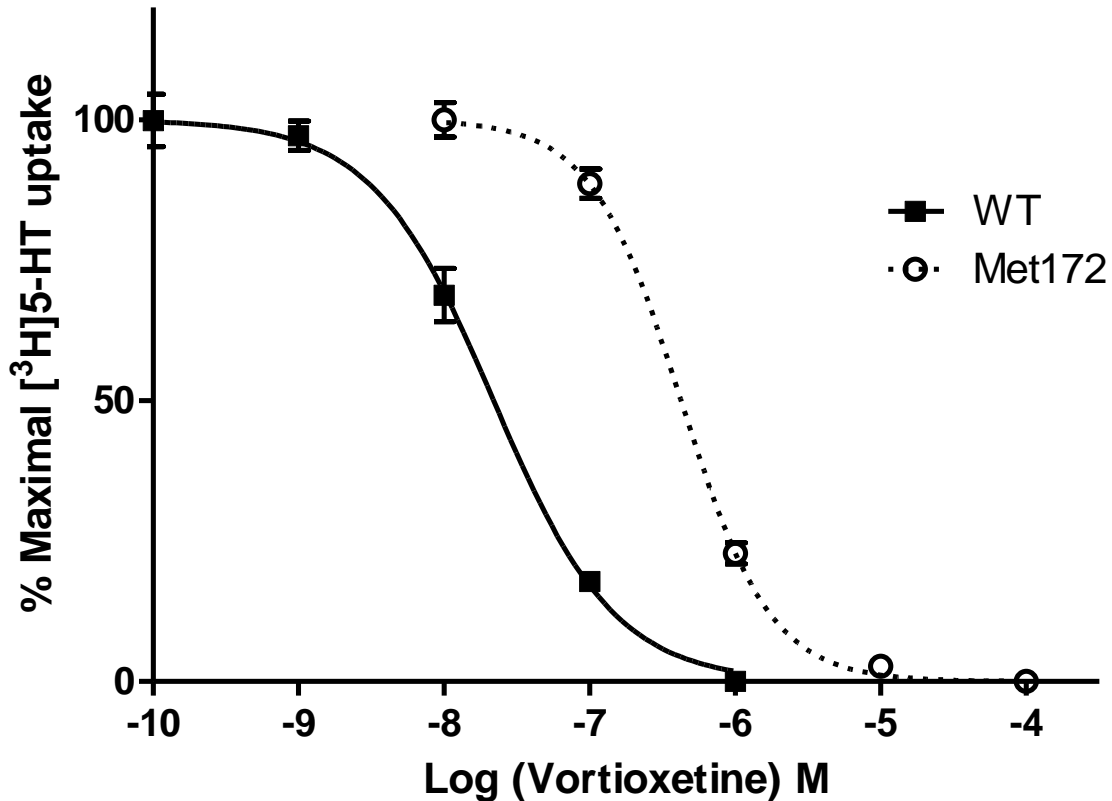


Figure 16. *In Vitro* Vortioxetine Competition 5-HT Uptake. Competition 5-HT uptake analysis in HEK-293T cells transfected with pcDNA3 plasmids containing WT SERT or SERT Met172. Vortioxetine (WT: K_I 21.9nM \pm 1.1; Met172 K_I 425nM \pm 1.07) was assessed for their ability to compete with [³H]5-HT uptake. (n = 4/condition). Vortioxetine is less able to inhibit 5-HT uptake in cells transfected with SERT Met172, as opposed to WT SERT. This affinity reduction for SERT Met172 is on the order of ~20 fold decreased potency.

These data suggest that we should be able to achieve similar success with vortioxetine, and that the ‘dose window’ we see here is not too small in our pursuit to remove the SERT component from the actions of vortioxetine. Though, this system is slightly artificial given the *in vitro* nature as well as the transfection conditions, so it would be more suitable to test this pharmacological sensitivity profile of vortioxetine in more suitable native-like system (ie. *Ex vivo* synaptosomal 5-HT competition uptake) before any actual attempts towards analyzing the requirement of SERT for the actions of vortioxetine.

Ex vivo Synaptosomal Competition 5-HT Uptake

Method

Following sacrifice via rapid decapitation, the whole brains of 8-12 week old male mice were dissected on ice and used for synaptosomal preparations as described previously (Thompson *et al*, 2011). Briefly, midbrain sections were mechanically homogenized in 0.32M sucrose in 5mM HEPES buffer. This homogenate was subjected to a low gravity centrifugation (10 min, 4°C at 1,000x g) in order to remove large cellular components (eg. mitochondria, nuclei, and large membranes) from the reconstituted nerve terminals, classified as ‘synaptosomes’. The supernatant after this first spin contains the synaptosomes. The synaptosomes are then pelleted via a high gravity centrifugation (15 min, 4°C at 10,000x g). The pellet was resuspended in KRH buffer containing glucose (for synaptosomal viability throughout the preparation and assay), pargyline (MAOI, prevents enzymatic metabolism of 5-HT), and ascorbic acid (preservative, prevents spontaneous oxidation of 5-HT). Equal volumes of synaptosomes were incubated with 20 nM [³H]5-HT (PerkinElmer) and varying concentrations of vortioxetine and incubated at 37 °C for 10

minutes. Uptake was terminated via vacuum filtration through GF/B filter (Whatman, Pittsburgh, PA, USA) and three washes with ice cold 1x PBS buffer. Specific uptake was defined by subtracting uptake obtained in the presence of 1 μ M paroxetine. Competition curve was fit to a single site inhibition model (Graphpad Prism 6.0)

Results

In order to assess whether the antidepressant actions of vortioxetine require SERT antagonism, we first needed to verify that vortioxetine is sensitive to the I172M substitution. Utilizing [³H]5-HT uptake into midbrain derived synaptosomes, and competition with vortioxetine, we demonstrate that vortioxetine is 20 fold less potent at SERT Met172 compared to WT SERT (**Figure 17**). These data corroborate what was found utilizing *in vitro* cellular transfection and competition uptake assays. This shift is roughly equivalent to the shift in potency of fluoxetine in the same paradigm, which we have shown can still render separations in functional SERT antagonism between these two SERT variants, it should therefore be possible to attain functional SERT antagonism with a single *in vivo* dose of vortioxetine in WT SERT mice that renders inconsequential antagonism in SERT Met172 mice. These studies alone cannot confirm this possibility, so further steps must be taken to ensure a separation in SERT binding as well as antagonism, both in reconstituted and functional determinations of these effects.

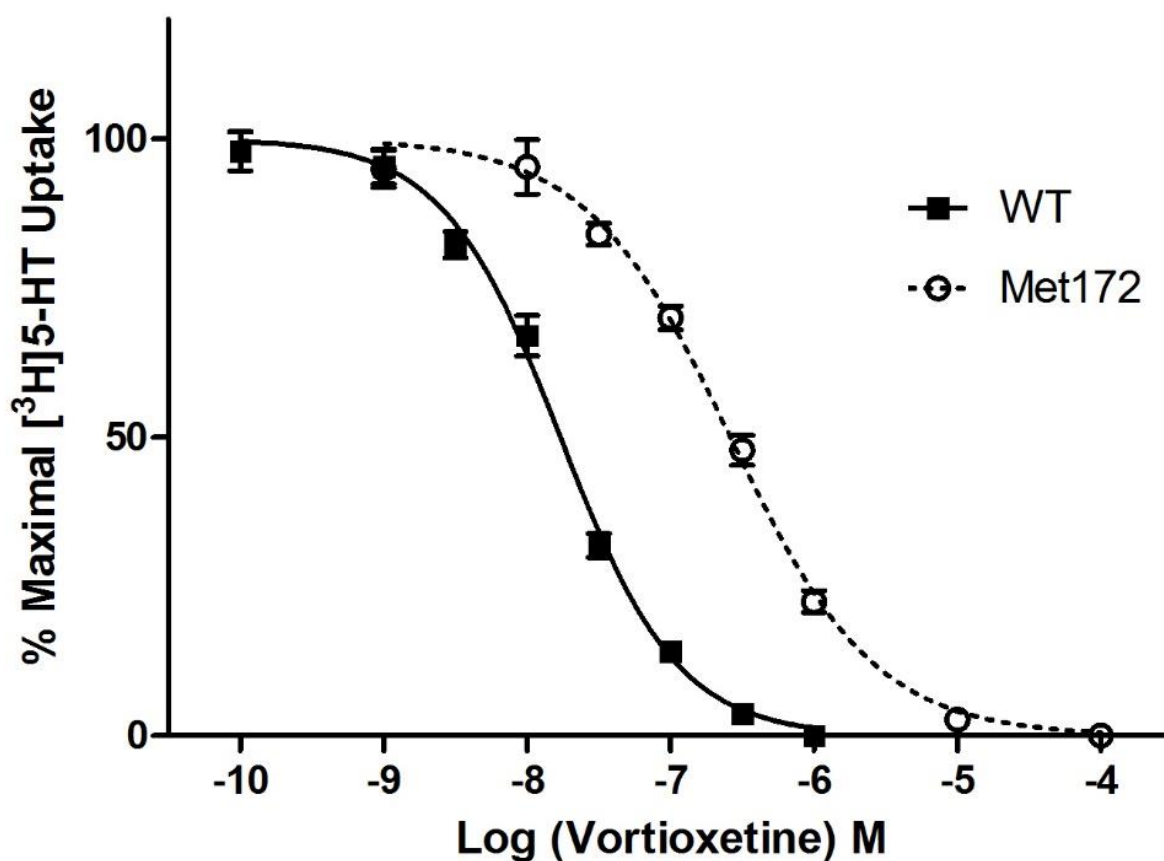


Figure 17. *Ex Vivo* Vortioxetine 5-HT Uptake Competition. The SERT Met172 mutant imposes altered pharmacological sensitivity with normal SERT expression and function. Competition 5-HT uptake analysis in midbrain derived synaptosomes. Vortioxetine (WT: K_i 17.1nM \pm 2.0; Met172: K_i 262nM \pm 2.0) was assessed for their ability to compete with [3 H]5-HT uptake. (n = 4/condition). Like the *in vitro* studies, vortioxetine displays reduced potency for SERT Met172 vs WT SERT (~20-fold reduction).

Ex Vivo SERT Occupancy

Method

We administered vortioxetine at 5 and 10 mg/kg i.p. to WT and SERT Met172 mice 1 hour prior to sacrifice. Following decapitation, brains were removed and frozen on dry ice and stored at -20°C until further use. Frontal cortex was coronally sliced at 20 µm thickness approximately 0.9-0.7mm anterior from Bregma (Paxinos and Franklin, 2004) via cryostat, and mounted onto slides. Slides were stored in slide boxes with desiccator pellets at -20°C until use. Slide boxes were allowed to defrost following 30 minutes of constant high flow air stream prior to opening. Slides were incubated in assay buffer (50 mM Tris HCl, 120 mM NaCl, and 5 mM KCl (pH=7.4)) containing 1nM [³H]paroxetine for 2h at room temperature. Non-specific binding was determined via incubation in excess (1 µM) non-radiolabeled paroxetine in assay buffer. Slides were then washed twice in assay buffer for 30 minutes, each at room temperature. Slides were allowed to air dry following washes for 30 minutes before transferal to a vacuum desiccator for at least 1 hour.

Autoradiographic analysis was then performed upon the slides using a Beta Imager (Biospace Lab, Paris, France). Bound radioactivity was measured by the Beta Imager for 24 hours, and quantified using the β-Vision software (Biospace lab, Paris, France). Surface radioactivity (measured as counts per min per millimeter squared, or cpm/mm²) was measured from a region of interest defined *a priori* based on the results of receptor mapping experiments using [³H]paroxetine, and included the lateral and medial septum, the nucleus accumbens, and the olfactory tubercle.

Total binding for a given mouse brain was determined by taking the average cpm/mm² from each of four replicate slices. Nonspecific binding was determined by averaging cpm/mm²

from the four brain slices in the nonspecific binding condition. Specific binding was determined for each mouse brain by subtracting the average nonspecific binding from total binding. Specific binding levels for each brain were then normalized to the average specific binding from the vehicle treated animals and expressed as a percent of average vehicle specific binding.

Fractional receptor occupancies were determined by subtracting these normalized values from 100. Fractional SERT occupancies observed in wild type or I172M mice were compared using a two way between subjects analysis of variance (ANOVA), with the factors being defined by genotype and dose. Where appropriate, *post hoc* analysis was conducted using Fisher's protected t. Alpha value stringency was set at $P = 0.05$.

Results

We aimed to validate that behaviorally relevant concentrations of vortioxetine would not fully saturate SERT Met172, which could limit the selectivity of our approach to selectively remove the SERT component of vortioxetine via the SERT Met172 model. The first way we chose to validate this idea was through brain slice competition binding. It was necessary to utilize radiolabeled and non-radiolabeled paroxetine as the competitive ligand due to its insensitivity to the I172M substitution. Pre-administration of the behaviorally relevant doses of 5 and 10 mg/kg vortioxetine (Guilloux *et al*, 2013) were able to produce robust SERT occupancy in WT animals, nearing and exceeding the requisite SERT occupancy for acute antidepressant efficacy of traditional pure SSRIs (Meyer *et al*, 2004). At both doses, SERT Met172 mice displayed significant reductions in vortioxetine SERT occupancy compared to WT mice (**Figure 18**).

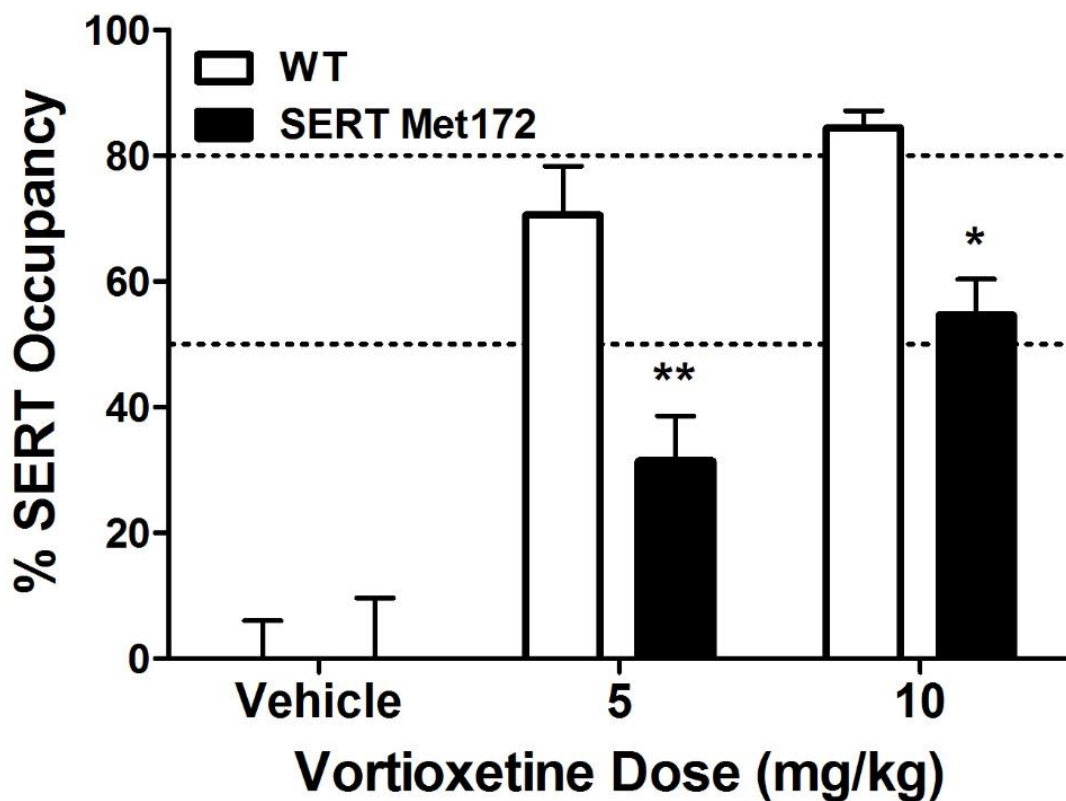


Figure 18. *Ex Vivo* Vortioxetine SERT Occupancy. Vortioxetine occupancy at SERT following peripheral administration of behaviorally relevant doses of vortioxetine. Coronal brain sections were harvested from mice that were administered 5 or 10 mg/kg vortioxetine (i.p.), which were sacrificed 1 hour following drug administration (the time by which behavioral analyses would occur in other studies).

However, the SERT Met172 mice did display appreciable levels of SERT occupancy, which could be problematic for our future studies. It should be noted, however, that both in *in vitro* and behavioral studies, classical SSRI occupancy at SERT must reach >80% occupancy to attain functional antagonism of SERT to yield inhibition of 5-HT uptake or antidepressant-like effects in the TST and FST (Meyer *et al*, 2004). So, it may not be problematic for our future experiments, as this lower level of vortioxetine occupation at SERT in the SERT Met172 animals may be inconsequential with respect to functional SERT antagonism. Though we cannot be sure of this given solely this assay, we must consider assays that are sensitive to functional SERT antagonism in a complete animal *in vivo* system.

In Vivo Chronoamperometry

Method

In vivo chronoamperometry with carbon fiber electrodes (30 μm diameter) was carried out according to the methods described previously (Thompson *et al*, 2011). The electrode–micropipette recording assembly was lowered into the CA3 region of the dorsal hippocampus [anteroposterior (AP), -1.94 from bregma; mediolateral (ML), $+2.0$ from midline; dorsoventral (DV) -2.0 from dura] of anesthetized mice. To assess 5-HT clearance kinetics, 5-HT was pressure ejected in increasing volumes to attain signal amplitudes matching *in vitro* calibration standards of approximately 0.5 to 10 μM . To examine the effect of vortioxetine on 5-HT clearance, exogenous 5-HT was intrahippocampally applied by pressure-ejection before and after peripheral (i.p.) injection of 10 mg/kg vortioxetine, and T_{80} values collected at 10 minute intervals.

Results

In order to assess whether the levels of SERT occupancy produce functional SERT antagonism *in vivo* in SERT Met172 mice, we first utilized *in vivo* chronoamperometry, which can measure real-time 5-HT clearance kinetics following exogenous administration of 5-HT (Daws *et al*, 1998; Schenk *et al*, 1983). This technique is sensitive to pre-administration of SSRIs, indicated by the delay in amount of time required to clear 80% of peak 5-HT signal (T_{80}), which is indicative of functional SERT antagonism (Baganz *et al*, 2008), and has been previously been validated to be sensitive to our SERT Met172 model (Thompson *et al*, 2011). Following peripheral administration of vortioxetine and measuring clearance kinetics every 10 minutes, we found significant elevations in T_{80} in WT mice across all time points compared to pre-drug baseline (**Figure 19**). SERT Met172, however, display no such increase in T_{80} following administration of vortioxetine, indicating that the levels of SERT occupancy that are produced in SERT Met172 animals are not functionally relevant and are unable to produce functional SERT-mediated 5-HT clearance in the hippocampus.

In Vivo Microdialysis

Method

For *in vivo* microdialysis, a guide cannula was implanted above the lateral hippocampus (stereotactic coordinates for the tip of the guide cannula were -3.18 A/P, 2.8 M/L, and -1 D/V, relative to bregma).

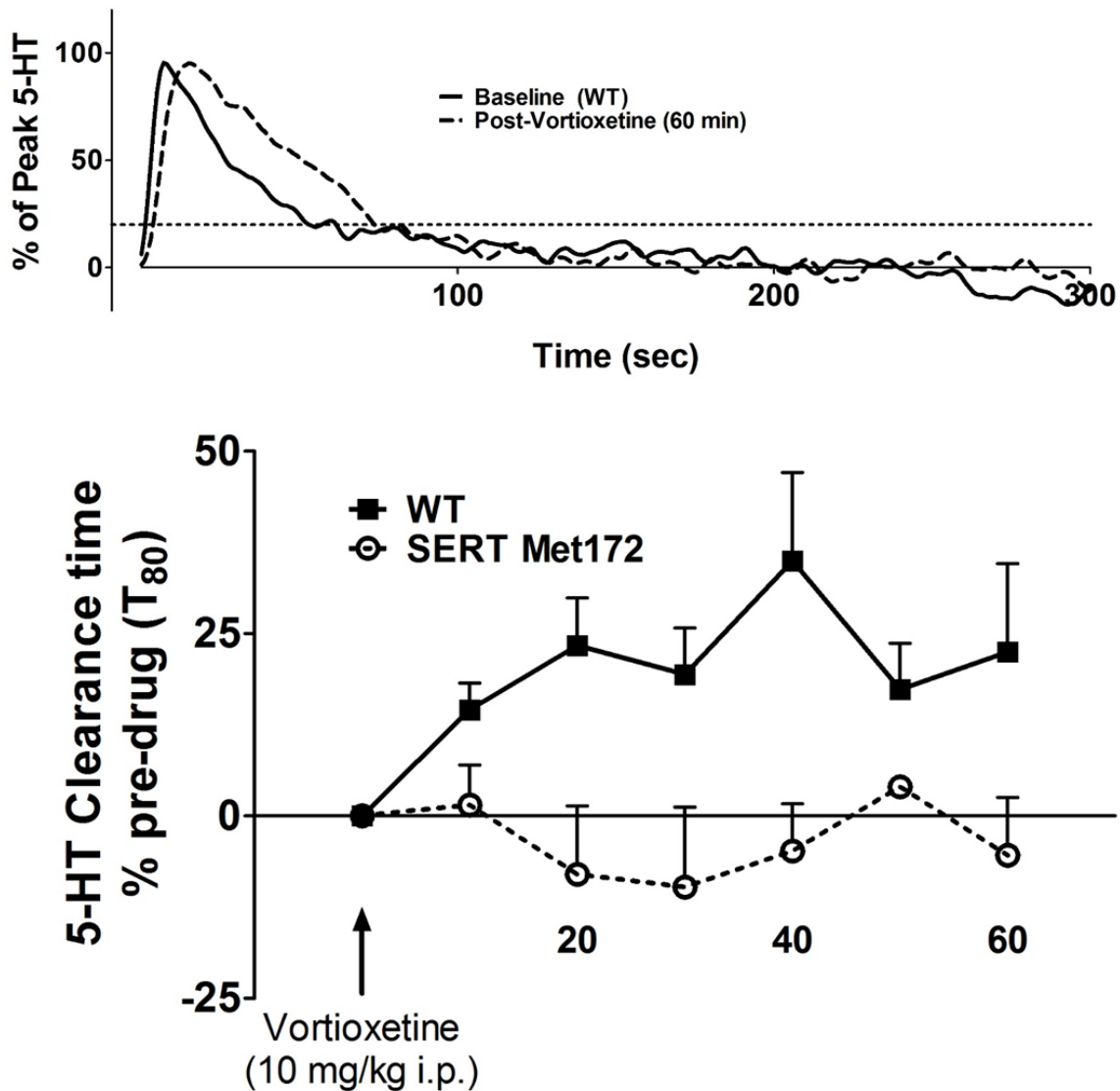


Figure 19. *In Vivo* Chronoamperometry of Vortioxetine-Inhibited 5-HT Clearance. Vortioxetine induced delayed clearance of 5-HT in dorsal hippocampus, detected via *in vivo* chronoamperometry. Extrasynaptic 5-HT content was measured from the CA3 region of the hippocampus in real time, via *in vivo* electrochemical detection. Vortioxetine (10 mg/kg, i.p) is able to delay 5-HT clearance in WT SERT but not SERT Met172, indicating functional SERT antagonism in WT but not SERT Met172 mice ($P < 0.05$, two way RMANOVA)

After recovery from surgery, the guide cannula was replaced with a microdialysis probe (3 mm active site, 20'000 Da cut-off, from Synaptech, MI) and perfused with aCSF (149 mM NaCl, 2.8 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgCl₂, pH 7.2) at a flow-rate of 1 μ L/min. aCSF samples were collected in 20 min intervals before and after 10 mg/kg vortioxetine (i.p.). 5-HT levels were quantified with HPLC/EC in the Vanderbilt Brain Institute Neurochemistry Core. Data were expressed as the level of 5-HT relative to pre-injection baseline. Probe location was verified post-mortem via coronal brain slice procedures (40 μ m). Data were analyzed via two-way RMANOVA, alpha stringency set to P = 0.05.

Results

Because *in vivo* chronoamperometry requires exogenous application of 5-HT, we felt it necessary to ensure that a behaviorally relevant dose of vortioxetine is incapable of producing functional SERT antagonism of endogenous 5-HT clearance in SERT Met172 mice. We thusly chose to explore this effect utilizing *in vivo* microdialysis. In this setup, behaviorally relevant doses of SSRIs administered are peripherally, leading to functional antagonism of SERT, elevating extracellular 5-HT in the extra-synaptic space, where it can be sampled with a microdialysis probe. Using this approach, we showed that SSRIs are unable to functionally antagonize SERT Met172, and sustain normal levels of 5-HT (Thompson *et al*, 2011). As shown here, vortioxetine can produce elevated levels of extracellular 5-HT in WT mice, indicative of functional SERT antagonism (**Figure 20**). Vortioxetine fails to stimulate extracellular levels of 5-HT in SERT Met172 mice (**Figure 20**), indicating that the highest concentration of vortioxetine we utilize in the behavioral studies is unable to functionally antagonize the 5-HT clearance by SERT Met172.

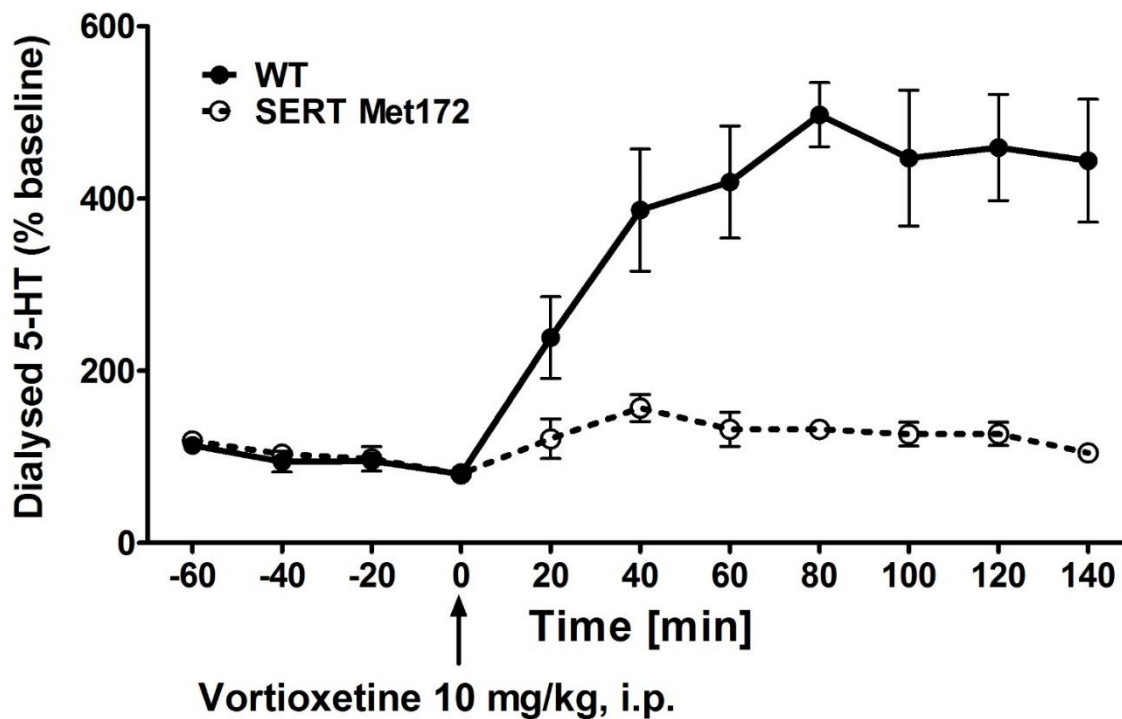


Figure 20. *In Vivo* Microdialysis of Vortioxetine-Induced Elevations in Extrasynaptic 5-HT. Vortioxetine induced increases in extrasynaptic 5-HT in dorsal hippocampus, detected via *in vivo* microdialysis. Dialysate was sampled from the CA3 region of the hippocampus every 20 minutes, and assessed for 5-HT content, via electrochemical detection. Vortioxetine (10 mg/kg, i.p) is able to elevate extrasynaptic 5-HT levels in WT SERT but not SERT Met172, indicating functional SERT antagonism in WT but not SERT Met172 mice ($P < 0.05$, two way RMANOVA)

Acute Behavioral Paradigm Animal Procedure

All behavioral assays were performed upon behaviorally naïve 8-12 week old male mice in the Neurobehavioral Core lab of the Vanderbilt University Medical Center. Animals were housed with a 12:12 hour light:dark cycle (light cycle: 7:00 to 19:00 hours), and all experimentation occurred between 13:00 and 18:00 hours. Animals were transferred to housing within the facility and allowed to acclimate for at least 1 week prior to behavioral manipulations. For acute drug studies, animals were acclimated to handling the day prior to experimentation, at which point body weight was measured to establish appropriate drug dose. All drugs were prepared fresh and dissolved in 10% β -cyclodextrin/0.9% sterile saline solution and injected intraperitoneal at 10 μ L/g body weight with either vehicle solution or 5 or 10 mg/kg vortioxetine. These doses were utilized to match prior studies by other research groups investigating the effects of vortioxetine in WT animals (Guilloux *et al*, 2013; Mørk *et al*, 2012). These prior studies, using WT C57Bl/6 mice, indicate high SERT occupancy was achieved at these doses as well as significant behavioral sensitivity. Our choice of these doses that reflects our desire to eliminate SERT occupancy in SERT Met172 mice to determine whether these doses maintain behavioral efficacy of vortioxetine without SERT antagonism. All experiments and data analyses were scored manually utilizing instantaneous sampling and binning methods of scoring while performed blind to drug condition and genotype.

Tail Suspension Test (TST)

Method

Mice were injected (i.p) with 10% β -cyclodextrin vehicle or vortioxetine (5 or 10 mg/kg) 60 minutes before a 6 minute TST. Mice were then suspended by taping the tail to a vertical aluminum bar and activity recorded by video. The apparatus allows for multiple mice to be tested at the same time. Mice are visually isolated from each other in 5 sided opaque plexiglass boxes, with the open side facing towards the room for video capture and handling purposes. Sixty minutes after injection, mice were then suspended by taping the tail to a vertical aluminum bar and activity was recorded by video. Immobility was defined when mice are motionless, excluding minute limb movements. Time immobile was manually assessed by an observer blinded to genotype and drug treatment. Data were analyzed following planned Student t comparisons, alpha stringency of $P = 0.05$.

Results

Following the indications reported above that we can selectively remove the SERT antagonism from the pharmacological actions of vortioxetine, but maintain its actions at 5-HT receptors, we aimed to probe whether vortioxetine was still behaviorally efficacious in the TST in SERT Met172 mice. As expected, WT animals in the TST display significantly increased time mobile relative to vehicle condition in response to both doses of acute vortioxetine and the SSRI paroxetine (**Figure 21**). SERT Met172 mice also display significant increase in mobility in response to the active SSRI paroxetine, which is insensitive to the Met172 substitution. Interestingly, the SERT Met172 mice displayed significant increases in mobility following both doses of vortioxetine (**Figure 21**).

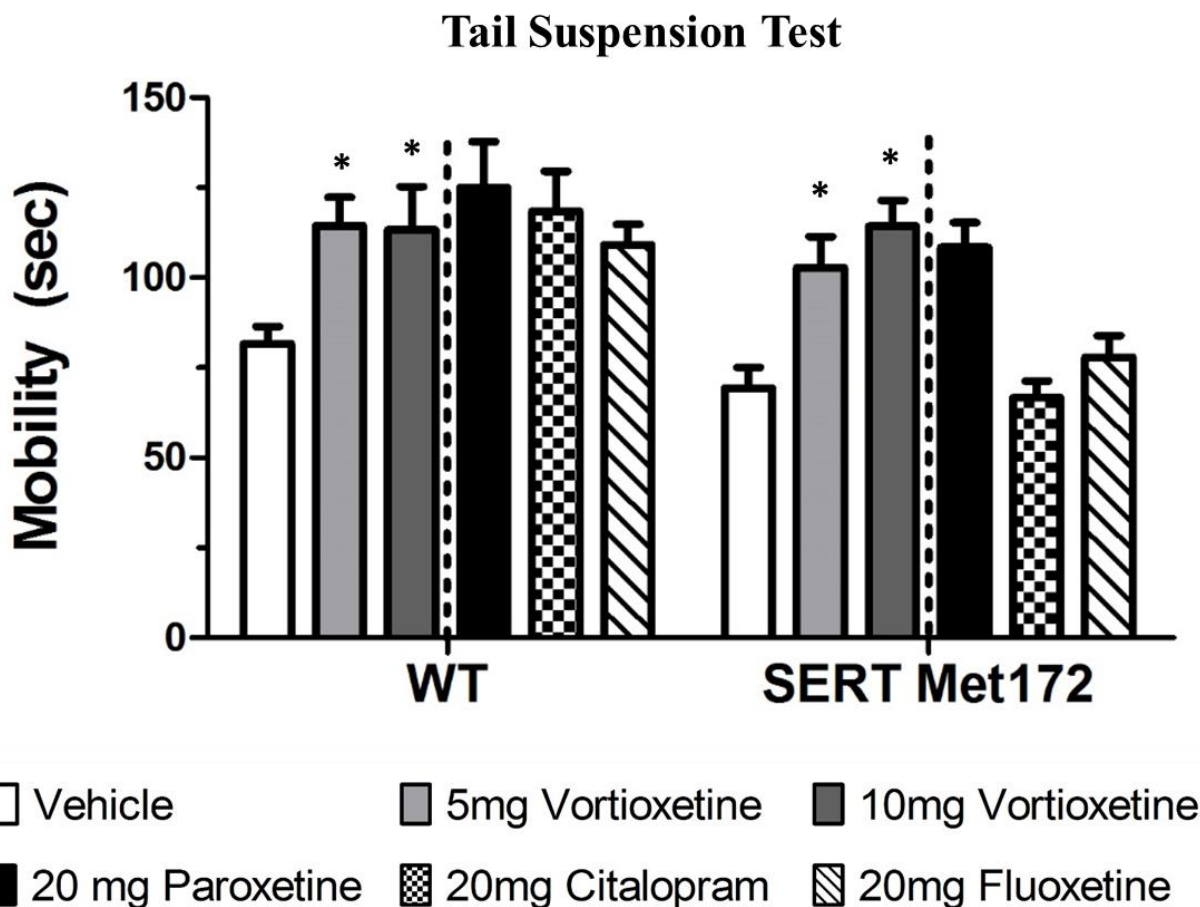


Figure 21. Tail Suspension Test with Vortioxetine. Actions of vortioxetine in the TST arise independently of SERT antagonism. All tests were performed 30 min after i.p. injection of 20mg/kg drug. Time mobile in a 6-minute TST. Two-way ANOVA revealed significant main effects of genotype. WT and Met172 mice display significant increases in mobility time in response to both doses of vortioxetine. Replotted are data from other experiments indicating the loss of efficacy of pure SSRIs citalopram and fluoxetine, yet maintained efficacy of paroxetine in Met172 mice. * indicates significance ($P < .05$) compared to vehicle treatment via planned students T test posttests ($n = 10-12$ per genotype and condition).

Forced Swim Test (FST)

Method

Mice were injected (i.p) with 10% β -cyclodextrin vehicle or vortioxetine 60 minutes before a 6-minute FST. Mice were placed in the center of a 15 cm diameter clear plexiglass cylinder filled with tap water (25-27 °C) to a depth of approximately 15 cm for the 6 minute FST and activity was recorded by video. Multiple mice were assessed at the same time, though, as with the TST, mice were kept visually unaware of the other mice via black plastic separators. Immobility was defined when mice only make movements to maintain balance. Time immobile was manually tabulated by an observer blinded to genotype and drug treatment. Data were analyzed following planned Student t comparisons, alpha stringency of $P = 0.05$.

Result

Given that we can remove the SERT antagonism from the pharmacological actions of vortioxetine, but maintain its actions at 5-HT receptors, we aimed to probe whether vortioxetine was still behaviorally efficacious in the FST in SERT Met172 mice. As expected, WT animals in the FST display significantly increased time mobile relative to vehicle condition in response to both doses of acute vortioxetine and the SSRI paroxetine (**Figure 22**). Paralleling the maintained efficacy of vortioxetine in the TST, SERT Met172 mice show significant increases in mobility following treatment with both doses of vortioxetine and paroxetine (**Figure 22**). Vortioxetine has been shown to induce climbing behavior in WT mice (Guilloux *et al*, 2013), which is thought to be due to its ability to enhance norepinephrine release in the prefrontal cortex (Mørk *et al*, 2012) via 5-HT₃ mediated silencing of forebrain cortical GABAergic neurons (Puig *et al*, 2004).

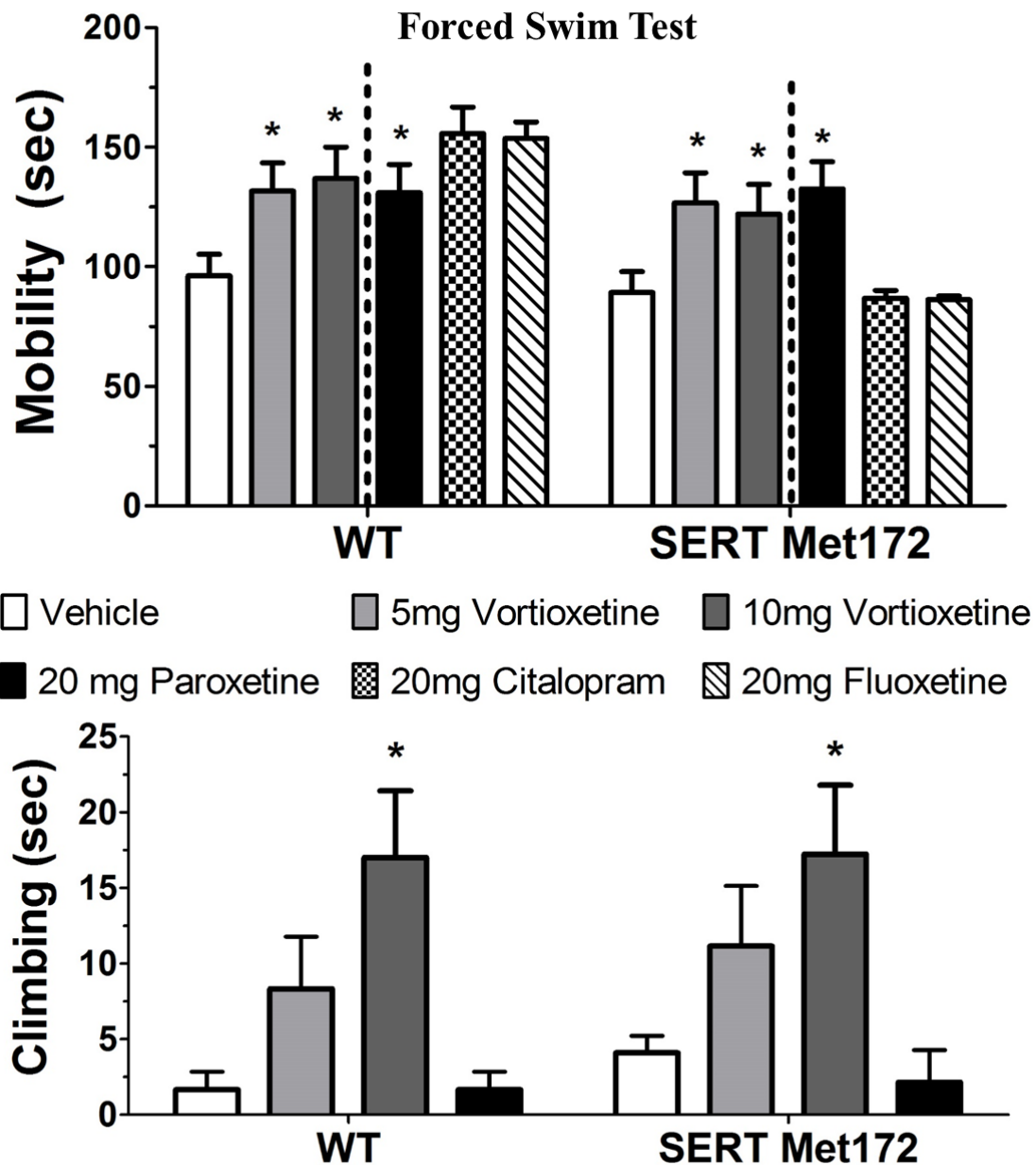


Figure 22. Forced Swim Test with Vortioxetine. Actions of vortioxetine in the FST arise independently of SERT antagonism. All tests were performed 30 min after i.p. injection of 20mg/kg drug. Time mobile and climbing in a 6-minute FST. Two-way ANOVA revealed significant main effects of genotype. WT and Met172 mice display significant increases in mobility time in response to both doses of vortioxetine. Replotted are data from other experiments indicating the loss of efficacy of pure SSRIs citalopram and fluoxetine, yet maintained efficacy of paroxetine in Met172 mice. Also shown is the SERT independent dose dependent increase in vortioxetine induced climbing behavior in both WT and SERT Met172 mice. * indicates significance ($P < .05$) compared to vehicle treatment via planned student's T test posttests ($n = 10-12$ per genotype and condition).

We observed this climbing-enhancing effect in our WT mice, in that vortioxetine could dose dependently increase climbing behavior in the FST (**Figure 22**), a trait that SSRIs like paroxetine do not possess (Detke *et al*, 1995). SERT Met172 mice also display increased climbing behavior following acute vortioxetine administration, but not after paroxetine (**Figure 22**). Together, these findings indicate that despite the loss of SERT antagonism, vortioxetine is still able to produce mobility enhancing effects in the TST and FST.

Novelty Induced Hypophagia (NIH)

Method

Animals were trained to consume a palatable substance (Vanilla Ensure®) in their home cage under low red light conditions (~50 lumens) in the testing room for a total of 3 days of 30 min sessions. On the first day of testing, mice were moved to a novel cage with no bedding and high white light illumination (~1200 lumens), where the latency to first consume Vanilla Ensure®, as well as the amount consumed after 30 min (in grams), were measured. On the following day, latency and consumption values of Vanilla Ensure® were assessed in the home cage under low light. To avoid ordering effects, the two testing days were switched for half of the mice. We administered vortioxetine in specially formulated rodent chow (to accomplish a ~10 mg/kg-day dosing regimen) for 28 days prior to behavioral screening, (David *et al*, 2009; Santarelli *et al*, 2003; Warner-Schmidt *et al*, 2011). Drug dosage was chosen for continuity with the acute drug sensitivity studies described earlier, as well as to produce serum concentrations of drug that are on the high end of clinically relevant serum concentrations in humans for traditional SSRIs (Dulawa

and Hen, 2005). Animals were singly housed during training and testing phases. Data were analyzed using two-way ANOVA and Bonferroni post hoc tests (Graphpad Prism 6.0).

Results

As noted above, the FST and TST are tests with predictive validity for antidepressant efficacy. Here we sought to evaluate the SERT-dependency of vortioxetine actions in the SERT Met172 model using an assay that possess more construct validity, and is thus more likely to measure the neurobiological processes that are required for human clinical efficacy. Thus, we implemented the NIH test, a behavioral paradigm sensitive to chronic, but not acute, antidepressant administration (Dulawa and Hen, 2005). The NIH test involves monitoring the latency of animals to approach and consume a known palatable substance in a novel, stressful environment, where SSRIs reduce latency and enhance consumption after chronic but not acute administration (Dulawa and Hen, 2005). We administered vortioxetine in specially formulated chow for 28 days prior to behavioral screening. Following vortioxetine administration, WT mice displayed a reduced latency to consume Vanilla Ensure® in the novel cage compared to vehicle condition (**Figure 23**). SERT Met172 mice, also display reduced latency to consume the Vanilla Ensure® in the novel cage. These findings suggest that despite ablating the functional effects of vortioxetine at SERT via the SERT Met172 substitution, the actions of vortioxetine at serotonin receptors are sufficient to produce antidepressant-like activity in the NIH assay. In other words, the chronic antidepressant behavioral activity of vortioxetine in the NIH test does not require SERT antagonism.

We had suspected the potential possibility of SERT-independent antidepressant effects of vortioxetine prior to our initial efforts, due to the direct actions of vortioxetine upon the 5-HT_{1A} receptor (Mørk *et al*, 2012).

Novelty Induced Hypophagia

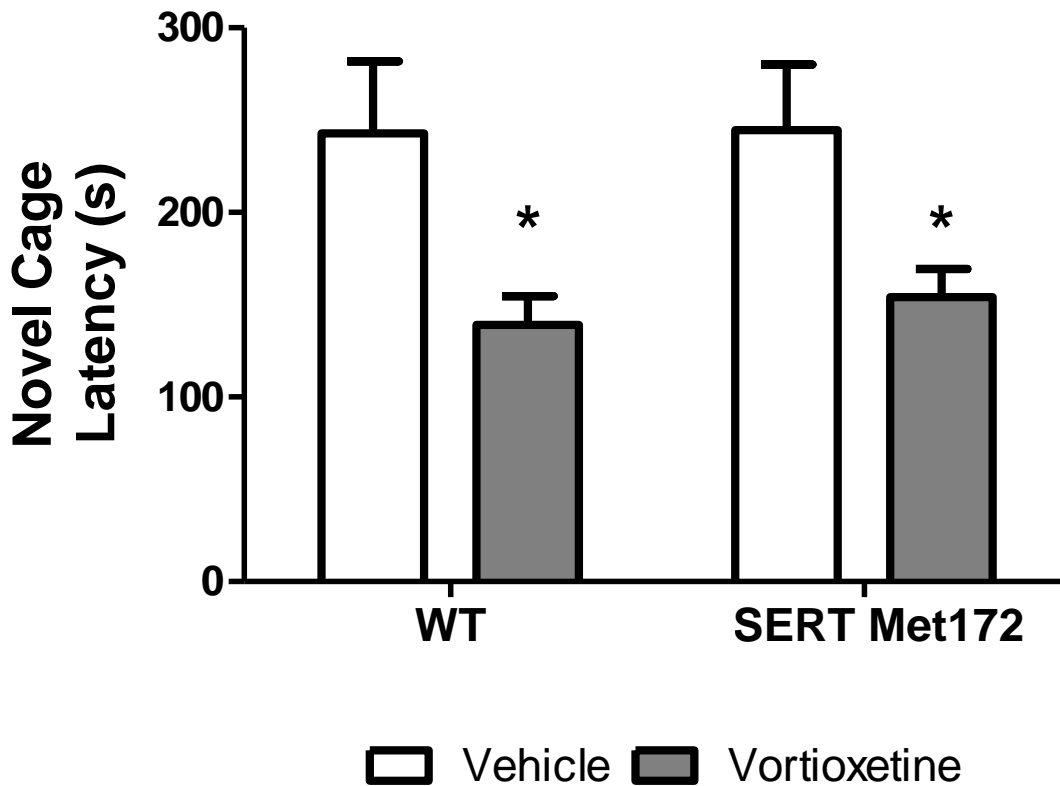


Figure 23. Novelty Induced Hypophagia with Vortioxetine. SERT antagonism is not required for the chronic effects of vortioxetine in the NIH test. Latency to consume Ensure® in novel cage was recorded. Two-way ANOVA revealed significant main effects of genotype. WT and Met172 mice display significant reductions in latency following chronic SSRI administration. * indicates significance ($P < .05$) compared to vehicle treatment following Two Way ANOVA and Bonferroni post-hoc tests ($n = 20-25$ per genotype and condition). Two Way ANOVA revealed significant main effect of drug ($F(1,78) = 13.26, P < 0.05$), but not of genotype ($F(1,78) = 0.10, P > 0.05$) or interaction ($F(1,78) = 0.06, P > 0.05$).

5-HT_{1A} activation has also been shown to stimulate hippocampal stem cell proliferation, as opposed to survival (Benninghoff *et al*, 2012; Klempin *et al*, 2010). 5-HT_{1A} receptors has been shown to be implicated in chronic behavioral and biochemical changes observed with SSRIs in the NIH assay, as well as hippocampal stem cell proliferation (Santarelli *et al*, 2003), a process that is required for chronic behavioral efficacy of SSRIs in the NIH test. Based upon these prior observations, we hypothesized that vortioxetine would show SERT-independent behavioral effects in the NIH by showing reduced latency to consume Vanilla Ensure® in the novel cage. These predictions were realized, provided the preserved efficacy of vortioxetine in the SERT Met172 mice. It would be interesting to compare the effect size of latency suppression between WT and SERT Met172 mice, as to ascertain whether SERT antagonism provides greater behavioral efficacy, though the assay was not powered to test this question. In our experiments, we could detect no differences in the magnitude of the effect sizes between the two groups, suggesting that SERT antagonism is rather inconsequential with respect to the actions of vortioxetine. Though one must also consider the limits in detection, and possible maximum effect that may be limiting this line of questioning. The NIH test provides sufficient dynamic range to detect antidepressant sensitive effects (Dulawa and Hen, 2005; Santarelli *et al*, 2003), though the magnitude is largely dictated by the strength of the anxiogenic paradigm and not perfectly suited for the separation of antidepressant strength (Dulawa and Hen, 2005). We cannot be sure which receptor, or set of receptors, is required for the activity of vortioxetine in this assay, though, reasonably, we can state that chronic vortioxetine does not require SERT antagonism for efficacy in the NIH test, and that, likely, its residual actions on 5-HT receptors are sufficient to produce the behavioral efficacy of vortioxetine in the NIH test.

Hippocampal Neurogenesis

Method

Proliferation: Following chronic administration of SSRIs and behavioral screening in the NIH test, mice were assayed for levels of hippocampal stem cell proliferation. Mice were administered 5-bromo-2'-deoxyuridine (BrdU; 150mg/kg i.p.; Sigma-Aldrich, St. Louis, MO, USA) to pulse label newly proliferating S-phase mitotic cells. 24hr following the last injection of BrdU, mice were anesthetized via injection of 100 mg/kg i.p. pentobarbital and transcardially perfused with ice-cold PBS, followed by ice-cold 4% paraformaldehyde. Brains were sectioned (40 µm) via freezing stage sliding microtome (Leica, SM2000R, Buffalo Grove, IL, USA). Every sixth section of the hippocampus (plates 41-61 (Paxinos and Franklin, 2004)) was immunostained for BrdU incorporation (mouse anti-BrdU; 1:1000; BD#347580; BD Biosciences; Franklin Lakes, NJ, USA) and detected following secondary antibody incubation (biotinylated donkey anti-mouse; 1:500; PA1-28627; ThermoFisher), ABC amplification (VectaStain; Vector Labs; Burlingame, CA, USA), and diaminobenzidine (DAB) detection. Brightfield stitched images were captured (Zeiss Axio Imager.M2) and stored for analysis. BrdU+ cells in the subgranular zone (SGZ) of the hippocampus were counted using the ITCN (Image-based Tool for Counting Nuclei) plugin for ImageJ (Byun *et al*, 2006) by an observer blinded to genotype and drug treatment. Total counts were extrapolated to whole hippocampus, accounting for the harmonic mean of hippocampal sections per mouse and initial sampling limits.

Survival: We injected a separate cohort with BrdU prior to administration of SSRIs. Proliferating cells at steady state were pulse labeled with BrdU (150mg/kg i.p.), and then their drinking water was then supplanted with SSRI infused drinking water (at 160 mg/L). After 4 weeks

of SSRI administration, mice were sacrificed and brain tissue collected and developed using the immunohistochemistry procedures described above. BrdU+ cell quantification for proliferation measures included the SGZ and the granule cell layer of the dentate. Cell counting was performed as described above. Data were analyzed using two-way ANOVA and Bonferroni post hoc tests (Graphpad Prism 6.0).

Result

Proliferation: Hippocampal neurogenesis is known to be stimulated after chronic, but not acute, antidepressant administration, which occurs along similar timescales of chronic SSRI behavioral sensitivity. Through the use of irradiation ablation studies, hippocampal neurogenesis has been shown to be required for the behavioral efficacy of chronic SSRI administration in the NIH test (Santarelli *et al*, 2003). SSRIs promote both hippocampal stem cell proliferation rate and survival of newly generated hippocampal stem cells (Wang *et al*, 2008), of which vortioxetine is no different (Guilloux *et al*, 2013). Consistent with these studies, chronic administration of vortioxetine stimulated the proliferation rate (**Figure 24**) of hippocampal stem cells in WT mice, assessed via BrdU+ immunohistochemistry. Vortioxetine was also able to stimulate the proliferation rate of these hippocampal stem cells in SERT Met172 mice (**Figure 24**), which agrees with prior studies that hippocampal stem cell proliferation stimulation tracks with behavioral efficacy in the NIH test.

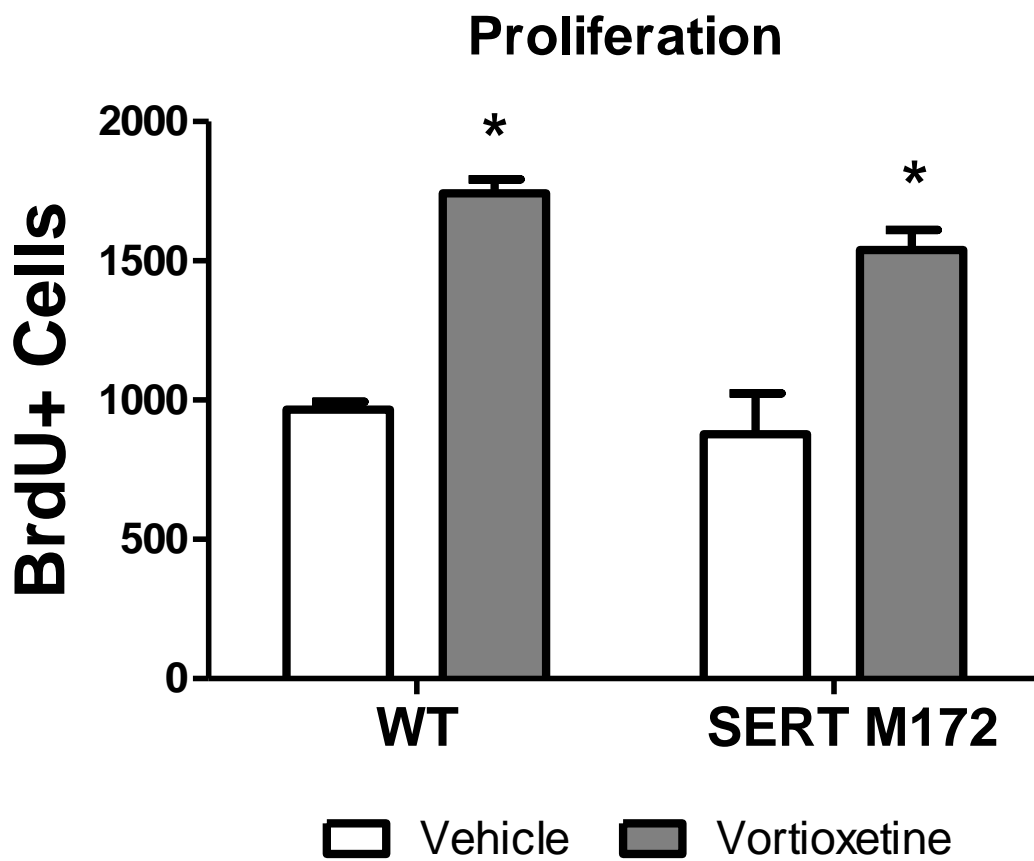


Figure 24. Hippocampal Neurogenesis: Proliferation Stimulation by Vortioxetine. SERT antagonism is not required for the stimulation of hippocampal stem cell proliferation and survival following chronic vortioxetine administration. Proliferation: Following administration of SSRIs, WT and Met172 mice display significant increases in stem cell proliferation rate. * indicates significance compared to vehicle ($P < .05$) following Two Way ANOVA Bonferroni post-hoc tests ($n = 4$ per genotype and condition). Two way ANOVA revealed insignificant effects of genotype ($F(1,10) = 3.20, P > 0.05$), significant effects of drug ($F(1,10) = 78.29, P < 0.05$), and insignificant interaction ($F(1,10) = 0.50, P < 0.05$).

These data corroborate our findings that vortioxetine can sufficiently stimulate hippocampal stem cell proliferation due to the SERT-independent actions of vortioxetine, potentially via 5-HT_{1A} receptor activation, given that 5-HT_{1A} receptor activation leads to the proliferation of these stem cells (Benninghoff *et al*, 2012; Klempin *et al*, 2010) and is required for both proliferation and efficacy of chronic SSRI behavioral efficacy in the NIH test (Santarelli *et al*, 2003). These data also indicate that SERT antagonism is not required for the proliferative component of hippocampal neurogenesis induced by chronic vortioxetine. Our findings support the potential for directly engaging specific 5-HT receptors as opposed to simply invoking SERT antagonism for the development of future antidepressant drugs.

Survival: After the neural progenitor cells proliferate and divide in the subgranular zone, they can differentiate into neurons and their functional integration and dendritic arborization into the hippocampus (van Praag *et al*, 2002). Most of these proliferated cells, however, do not survive, and the pruning of proliferated cells is thought to be under the control of 5-HT receptors (Klempin *et al*, 2010). The major effect of chronic SSRIs for behavioral efficacy in the NIH test is their stimulatory effect upon the stem cell proliferation, though they can also promote the survival of basally proliferated stem cells (Klempin *et al*, 2010). The function of the hippocampal neurogenesis, both proliferation and the survival/integration processes, is not fully understood, but has been implicated to maintain hippocampus-dependent behavioral plasticities (Benninghoff *et al*, 2012). In this aim, we tested to see the contribution of vortioxetine towards promoting the survival of basally proliferated hippocampal stem cells, and whether this process required SERT antagonism. Following a pulse label of basally proliferating hippocampal stem cells with BrdU, we administered vortioxetine to WT and SERT Met172 animals for 4 weeks. Following the immunohistochemistry for the BrdU label, we found that both WT and SERT Met172 mice display

increased rates of survival (**Figure 25**). These data indicate that stem cell survival component of vortioxetine, as well as stem cell proliferation, does not require SERT antagonism, and that its array of activity at other targets, specifically 5-HT receptors, is sufficient to stem cell proliferation and survival.

Conclusions

The rationale for the use and development of vortioxetine in the clinic stems from the broad and engagement of multiple 5-HT receptors following SSRI administration. With SSRI administration and subsequent SERT blockade, all 5-HT receptors are stimulated and activated due to this generic enhancement of serotonergic signaling, some of which might be deleterious to the desired antidepressant profile (Ramamoorthy *et al*, 2008; Wesołowska *et al*, 2006). It could thus be beneficial to develop pharmacological agents that can selectively target specific 5-HT receptors, limiting actions at receptors that diminish efficacy or those that produce unacceptable side effects, all while possibly eliminating traditional engagement at SERT. Vortioxetine aims to accomplish this aim of specific 5-HT receptor engagement through its actions as an agonist at 5-HT_{1A} and as a partial agonist at 5-HT_{1B}. Vortioxetine is also an antagonist at 5-HT₃ and 5-HT₇ receptors, and retains significant activity as a SERT antagonist (Mørk *et al*, 2012), though whether its activity at SERT is relevant for clinical efficacy has not been tested.

Due to preclinical studies indicating potential antidepressant utility of compounds that target 5-HT receptors that vortioxetine targets individually, our lab sought to query whether these SERT-independent 5-HT receptor actions of vortioxetine could be sufficient to produce antidepressant effects.

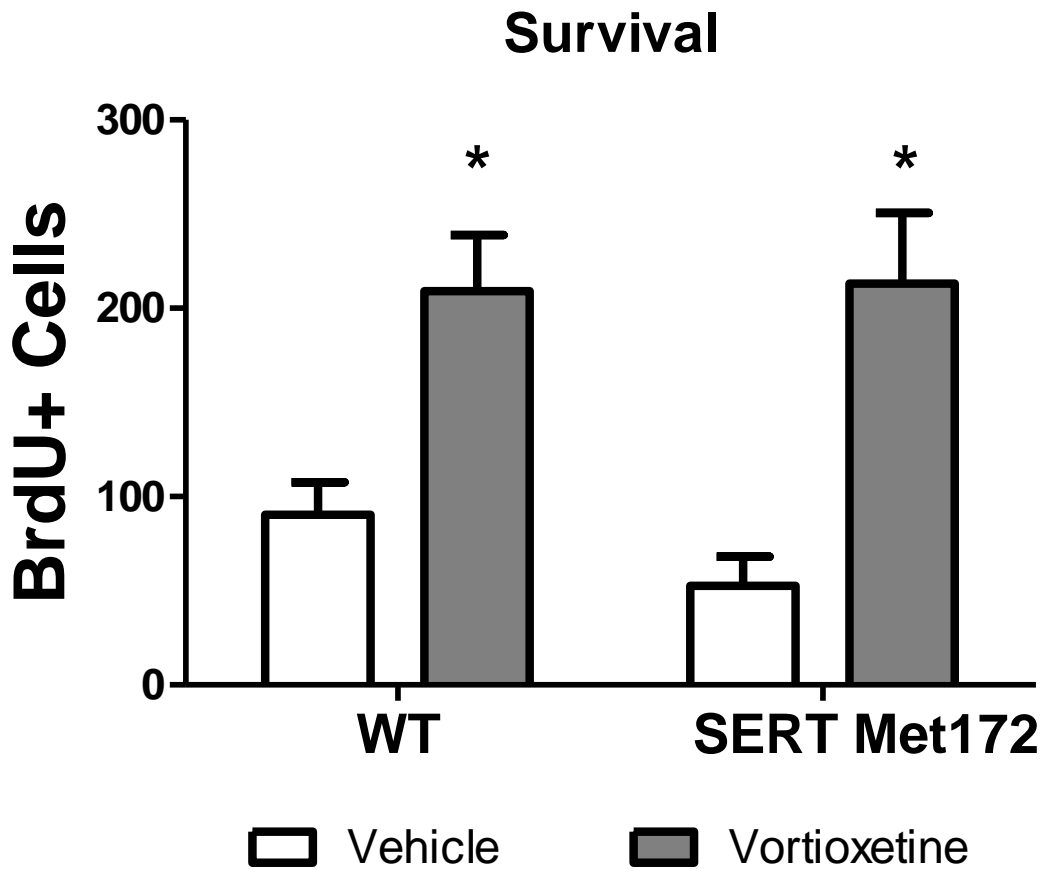


Figure 25. Hippocampal Neurogenesis: Survival Preservation with Vortioxetine. SERT antagonism is not required for the survival of basally proliferated hippocampal stem cells following chronic vortioxetine administration. Survival: Following administration of BrdU, vortioxetine was administered to WT and SERT Met172 mice. WT and Met172 mice display significant increases in survival of stem cells. * indicates significance compared to vehicle ($P < .05$) following Two Way ANOVA and Bonferroni post-hoc tests ($n = 4$ per genotype and condition). Two Way ANOVA revealed insignificant main effect of genotype ($F(1,9) = 0.37, P > 0.05$), significant effect of drug ($F(1,9) = 25.08, P < 0.05$), and insignificant interaction ($F(1,9) = 0.56, P > 0.05$)

In order to accomplish this task, we utilized the SERT Met172 mouse model, to selectively reduce the ability of vortioxetine to bind and inhibit SERT, all while preserving SERT expression and function and thus free from the compensatory alterations that plague the SERT knockout model (Bengel *et al*, 1998; Cour *et al*, 2001; Fabre *et al*, 2000; Li *et al*, 2000; Lira *et al*, 2003; Rioux *et al*, 1999). Utilizing the SERT Met172 model, vortioxetine actions at these 5-HT receptors are preserved while limiting SERT antagonism. In this aim, I tested whether vortioxetine retained antidepressant properties in acute and chronic models of antidepressant efficacy. In the acute tests for antidepressant sensitivity, vortioxetine is able to reduce immobility time equivalently in both WT and SERT Met172 mice in both the TST and FST, indicative of an antidepressant-like effect. Interestingly, vortioxetine is able to produce a climbing effect in the FST in both WT and SERT Met172 mice, an effect that is not produced by SSRIs and other SERT blockers and that is usually seen in norepinephrine enhancing antidepressants, such as NET blockers, though vortioxetine has no significant activity at norepinephrine receptors or NET (Mørk *et al*, 2012). This effect is thought to be due to the antagonist effect of vortioxetine at 5-HT₃ receptors (Puig *et al*, 2004). The relevant antagonism of 5-HT₃ receptors, which are thought reside on GABAergic interneurons in the PFC, would effectively reduce the GABAergic inhibition of norepinephrine release in the PFC, thereby driving this climbing effect in the FST (Puig *et al*, 2004). This interesting effect further exemplifies the SERT independence of vortioxetine in acute antidepressant-sensitive assays. Together, our findings reveal that vortioxetine does not require SERT for its antidepressant activity in assays that are sensitive to acute antidepressant administration.

As has been noted previously, these acute measures of antidepressant efficacy are not ostensibly measuring the same biological processes that arise during the timecourse of clinical antidepressant efficacy, which requires weeks of chronic administration. Moreover, many

preclinical compounds that have shown antidepressant-like efficacy in the TST and FST, have failed in clinical trials or failed to surpass antidepressant efficacy of SSRIs. Therefore, there is no guarantee that the SERT independence of vortioxetine in the ability to produce antidepressant-like effects in the TST and FST would translate to chronic antidepressant sensitive models. Alternatively, the actions of vortioxetine at 5-HT receptors could merely act in an accessory manner, dependent upon that overall enhancement of serotonergic tone brought about by SERT inhibition. However, 5-HT_{1A} stimulation has been shown to drive hippocampal stem cell proliferation (Klempin *et al*, 2010), and the presence of the receptor is required for the stimulatory effects on hippocampal stem cell proliferation and chronic antidepressant sensitivity (Santarelli *et al*, 2003). Given the stimulatory actions of vortioxetine at 5-HT_{1A} receptors, vortioxetine may drive hippocampal stem cell proliferation and chronic antidepressant sensitivity, via a 5-HT receptor linked pathway as opposed to SERT antagonism.

I next tested the ability of vortioxetine to induce antidepressant-like effects in assays that are sensitive to chronic—but not acute—antidepressant administration. After administering vortioxetine chronically to WT and SERT Met172 mice, I then found that both WT and SERT Met172 mice were behaviorally responsive to the drug, with respect the anxiolytic properties of consumption latency values. These data indicate that SERT is not required for the chronic behavioral actions of vortioxetine, and that the combined 5-HT receptor activity of vortioxetine is sufficient to produce an antidepressant effect. Next, I tested the ability of vortioxetine to stimulate hippocampal stem cell proliferation, and whether this process required its ability to inhibit SERT. Using the same models as previously, both WT and SERT Met172 mice displayed significant increases in hippocampal stem cells following chronic vortioxetine administration, indicating

again that vortioxetine does not require activity at SERT for this effect and that the 5-HT receptor activity profile of vortioxetine outside of SERT binding is sufficient to produce this effect.

My findings show that vortioxetine does not require activity at SERT to produce antidepressant effects in models that are sensitive to either acute or chronic antidepressant administration. Although these data are limited in scope to the SERT-independent efficacy of vortioxetine, they demonstrate the principle that future iterations of serotonergic antidepressant drugs may not require SERT antagonism. The field of antidepressant pharmacology has largely stagnated surrounding the dogma that engagement at SERT is required for clinical efficacy. Elevations in 5-HT brought about by SERT antagonism produces global enhancement 5-HT receptor activation, which may be deleterious towards antidepressant profile, as well as enhancing side effect profile. Next generation serotonergic antidepressants would be wise to engage those 5-HT receptors that are beneficial to producing antidepressant effects while avoiding those that negate those effects. Vortioxetine is able to accomplish this aim, albeit with major activity at SERT. It is able to do so with a single chemical compound, which is remarkable and surprising in its own right, though I do not expect future drug development efforts to achieve this feat, nor should it absolutely be necessary to do so. These studies do provide justification for future development of multitargeted drugs at a preclinical level, and could usher in a new wave of serotonergic based antidepressants that lack appreciable activity at SERT, and representing a logical next step in the development of serotonergic antidepressants lacking SERT antagonism.

CHAPTER VI

CONCLUSIONS AND FUTURE EXPERIMENTATION

The purpose of these studies was multifaceted, all designed to assess the requirement of SSRIs to antagonize the serotonin transporter for its antidepressant effects. The complicated and delayed nature by which antidepressants exert their effects in the clinical population has generated some doubt to the serotonin hypothesis of depression, and there have been various examples that have directly questioned whether the antidepressant effects of SSRIs are enacted through SERT antagonism. Up until this point, there have been no definitive tests or models that have been able to address these concerns without significant caveats. The SERT knockout mouse, while intriguing to test the nature by which SERT is required for the antidepressant effects of SSRIs, exhibits major compensatory alterations that perturb normal neurodevelopment and serotonergic homeostasis, rendering an abnormal environment within which the SSRIs act. Though the studies using the SERT knockout model suggest that SSRIs do require SERT antagonism (Holmes *et al*, 2002), the confounds that plague the model have done little to dispel the multiple lines of research suggesting the role of non-SERT SSRI targets involved in the antidepressant effects of SSRI pharmacotherapy.

The SERT Met172 mouse model was developed in part to test this question of SERT dependence of SSRI antidepressant activity (Thompson *et al*, 2011). This effort was led by Dr. Randy Blakely, who sought to create a model that did not have the problems the SERT knockout model possesses, in part to test the SERT dependence of SSRI actions. The aim of this endeavor was to create a model that lacks pharmacological inhibition of SSRIs at SERT, but lacks the

compensatory alterations seen in the SERT knockout model. This effort led the group to pursue the observed differences in pharmacological sensitivity of different serotonin transporters across phylogeny to SSRIs (specifically human and mouse SERT compared to *Drosophila* SERT), and ascertain which amino acid residues were integral to that shift in pharmacological sensitivity. The result of much mutagenesis work was that Ile172 was highly responsible for high affinity SSRI binding at human and mouse SERT, whereas the corollary amino acid in *Drosophila* (methionine), when substituted into human or mouse SERT, could recapitulate the reductions in SSRI sensitivity seen in *Drosophila* SERT (Henry *et al*, 2006). DNA encoding this substitution (Ile172Met; I172M) was then achieved in mice via embryonic knock-in homologous recombination techniques. The substitution did not impact SERT protein expression, SERT surface expression, CNS biogenic amine levels, or SERT protein function (Thompson *et al*, 2011). Importantly, behaviorally relevant doses of SSRIs could not functionally antagonize SERT Met172 in *in vivo* paradigms (Thompson *et al*, 2011). The genetic background of the mouse in which the embryonic knock-in was created, however, was not conducive to further behavioral experimentation, including achieving the answers pertaining to whether SERT interactions are required for SSRI antidepressant activity. The importance of these efforts, as well as for the plans for this mouse moving forward, dictated that the SERT Met172 mouse be backcrossed onto a suitable genetic background. Here, we show the results of the studies on the SERT Met172 mouse following the backcrossing onto the C57Bl/6 background. I first ensured that the act of backcrossing did not perturb the observation that SERT Met172 is benign with respect to SERT protein expression, CNS biogenic amine levels, and SERT protein function, and induced these parameters equivalently to WT littermates (Nackenoff *et al*, 2015). Additionally, the expected loss of potency for SERT antagonists, fluoxetine and citalopram, were preserved in the C57Bl/6 SERT Met172 model, with maintained sensitivity for paroxetine,

which serves as an important positive control for our experiments (Nackenoff *et al*, 2015). Because of the utility of the C57Bl/6 mouse model for behavioral testing, we next moved to behavioral assays that are sensitive to acute administration of SSRIs. Should SSRIs retain behavioral activity in SERT Met172 mice, this would indicate that SSRIs do not require SERT antagonism, since behaviorally relevant doses of SSRIs do not functionally antagonize SERT Met172 and thus maintained CNS interactions with other non-SERT targets would be producing the antidepressant-like effects in these acute SSRI sensitive tests. When I tested these mice in the TST and FST, I found that SERT Met172 mice fail to respond to fluoxetine and citalopram, paralleling their inability to antagonize SERT Met172 *in vivo*. Thus, acute antidepressant effects of the SSRIs fluoxetine and citalopram in the preclinical forced swim and tail suspension tests require SERT antagonism.

Since acute preclinical tests of antidepressant sensitivity do not accurately reflect the complex nature of clinical response to antidepressants, we next wanted to move to assays that are sensitive to chronic—but not acute—SSRI administration. These tests would best to parallel the chronic nature of SSRI antidepressant administration that is required for clinical efficacy. In this aim, I tested the ability of chronic SSRI administration to induce antidepressant-like effects in the NIH test. SERT Met172 mice fail to respond behaviorally to chronic SSRI in this test (Nackenoff *et al*, 2015). Additionally, I also investigated the degree to which SSRIs can enhance hippocampal stem cell proliferation in the SERT Met172 mice, an effect that occurs with chronic SSRI administration, and is required for chronic SSRI behavioral sensitivity (Santarelli *et al*, 2003). Like the lack of sensitivity in the NIH test, following chronic administration of SSRI SERT Met172 mice fail to display enhanced hippocampal stem cell proliferation, nor do they display any increased rates of survival of basally proliferated stem cells (Nackenoff *et al*, 2015), both of which

are produced by chronic SSRI administration in WT mice. These data indicate that both acute and chronic antidepressant effects induced by SSRIs require SERT antagonism. These results are important in their own right, due the fact that they represent the most specific set of assays to date that could definitively state that SERT antagonism is required for the antidepressant efficacy of SSRIs.

The SERT Met172 mouse model was not created solely for this purpose, however. A significant advantage over the SERT knockout model is that while both models remove the pharmacological target of SSRIs [at behaviorally and clinically relevant doses], the SERT Met172 model is devoid of the compensatory alterations produced by the SERT knockout model. This is important when considering the future potential of the SERT Met172 model that cannot be achieved by the SERT knockout. Because of the multifaceted and temporally complex nature of the antidepressant effect seen clinically, much work has been done to isolate the key facets of the antidepressant response, which are obviously more complicated than simply SERT antagonism. Were we able to identify the necessary downstream biological alterations and responses to chronic SSRI administration that bring about clinical antidepressant efficacy, we could potentially directly aim to produce those necessary changes through new drug treatment strategies in order to produce new faster treatments for depression. Isolating these necessary changes, however, is difficult due to the nature by which SSRIs also engage other non-SERT targets. Any attempts to identify the genetic consequences of chronic SSRI administration in mice would generate datasets that would contain both SERT-specific changes in addition to those alterations brought about by engagement of SERT-independent targets. The SERT knockout model can be utilized, but as noted before, the compensatory alterations found in that model render an abnormal environment in which these SSRIs can act. And unlike behavior, biochemical alterations that arise with chronic administration

may be more sensitive to compensatory alterations, and any such findings would severely cloud the accuracy of perceived SERT-specific effects of SSRIs. The SERT Met172 mouse model represents the best control condition for the elucidation of SERT specific effects of chronic SSRI administration. When compared against WT mice, since SERT Met172 mice only remove the ability of SSRIs to bind and antagonize SERT, any molecular or cellular changes seen in SERT Met172 can only be due to non-SERT interactions of SSRIs and can be easily removed from WT datasets. Efforts to conduct those studies are currently underway, and soon we can start deconstructing the serotonin path towards antidepressant efficacy. Identifying the key essential elements on the serotonin dependent pathway of antidepressant efficacy can illuminate essential components in the antidepressant response, as well as identify potentially better targets of future antidepressants. Through prior studies, we know that SSRIs impact the expression of kinases (Rausch *et al*, 2002), BDNF and other neurotrophic elements (Alme *et al*, 2007; Castrén and Rantamäki, 2010), NMDA receptor subunit expression (Boyer *et al*, 1998), PKA pathway sensitive transcription factor CREB expression (Nibuya *et al*, 1996), as well as 5-HT receptor expression and sensitivity (Hensler, 2002), among many others. Whether these effects result from SERT antagonism is unknown. Our attention will focus on neurotrophic factors in the hippocampus, as neurogenesis seems to be a critical element in the antidepressant effect invoked by chronic SSRI administration, and may represent a future direct target for antidepressants, skipping SERT antagonism entirely (Malberg and Schechter, 2005). Though only through comparisons to the SERT Met172 model can we determine which of these are dependent upon enhancement in serotonergic tone and SERT antagonism.

These attempts represent a desire to further isolate the key facets of antidepressant actions and develop more efficient techniques by which we can induce antidepressant effects. In very early

antidepressant therapies, albeit serendipitous, monoamine oxidase was targeted. These drugs produced enhancements in serotonergic signal transmission via the inhibition of the metabolism by monoamine oxidase. This approach, though clinically effective, is not the most efficient or specific means to enhance serotonergic neurotransmission. It was the only antidepressant pharmacotherapy at the time, and with improvements in antidepressant pharmacology, MAOIs are rarely utilized today. Following in the footsteps of MAOIs, tricyclic antidepressants were introduced, again serendipitously. These TCAs also enhanced serotonergic neurotransmission via the inhibition of the serotonergic synaptic clearance, allowing for an increase in 5-HT signal duration. Though these TCAs are arguably as efficient as current SSRI pharmacotherapies at inhibiting SERT and prolonging 5-HT signal duration, TCAs were largely abandoned clinically due to their cardiotoxic side effects and drug-drug interactions limited therapeutic utility. SSRIs represent the current standard of care for antidepressant pharmacotherapy, though they are not necessarily the most efficient and technologically advanced considering what we know about the role of different serotonin receptors that contribute to, or retract from, the antidepressant effect. In fact, though engagement of certain serotonin receptors is beneficial to the antidepressant effect, activating others can potentially be deleterious to the desired antidepressant effect (Nakagawa *et al*, 1998; Singh and Lucki, 1993; Wesolowska *et al*, 2006). This oppositional and competing nature of serotonin receptor activation events may contribute to the delayed clinical efficacy of SSRI antidepressants. At the least, it represents a technological barrier that could easily be traversed were we to create antidepressants that engage desired 5-HT receptor activity and removes the deleterious effects at others.

The novel antidepressant vortioxetine aims to accomplish this technological achievement (Guilloux *et al*, 2013; Mørk *et al*, 2012). Vortioxetine, while engaging and antagonizing SERT,

binds and activates 5-HT1A and 5-HT1B while antagonizing 5-HT3 and 5-HT7 receptors (Mørk *et al*, 2012). The strategy for this compound aims to possess faster therapeutic efficacy and/or reduced side effect profile, as 5-HT3 activation induces nausea and there already exists a clinical compound that antagonizes the 5-HT3 receptor for the reduction in nausea (Glare *et al*, 2011). Vortioxetine, while invoking traditional antidepressant actions at SERT, may not require its activity at SERT given that the antidepressant effect can be recapitulated by directly targeting one or more of the receptor targets invoked by vortioxetine outside of SERT antagonism (Ramamoorthy *et al*, 2008; Robinson *et al*, 1990; Singh and Lucki, 1993; Tatarczyńska *et al*, 2005; Wesółowska *et al*, 2006). We wanted to query whether the antidepressant effect invoked by vortioxetine required SERT antagonism, as SERT-independent antidepressant effects would represent a technological advancement beyond pure SERT engaging antidepressants. In my studies, I tested the ability of vortioxetine to induce antidepressant-like effects in the SERT Met172 mouse model, using assays that are sensitive to acute and chronic SSRI administration. Firstly, I found that vortioxetine remains capable in inducing antidepressant-like effects in SERT Met172 mice in the TST and FST, tests that are sensitive to acute single dose administration of vortioxetine, indicating that vortioxetine does not require SERT antagonism for its ability to induce antidepressant-like effects. Additionally, vortioxetine is still capable of inducing climbing effects in the FST in SERT Met172 mice, an effect brought about not by SERT antagonism, illustrating further that vortioxetine acts in a SERT-independent manner, within certain endpoints. I then moved to analyze whether chronic antidepressant actions of vortioxetine required SERT antagonism. As acute antidepressant sensitive measures are not representative of clinically relevant biological mechanisms required for antidepressant efficacy, we needed to move to assays that are only sensitive to chronic—but not acute—antidepressant administration. For this, we chose the

NIH test and subsequently assessed hippocampal neurogenesis, a process that is required for SSRI efficacy in the NIH test (Santarelli *et al*, 2003). Chronic administration of vortioxetine can induce behavioral sensitivity in the NIH test as well as stimulate the production of hippocampal stem cells (Guilloux *et al*, 2013). Our aim was to investigate whether the removal of SERT from the pharmacological profile of vortioxetine, through the use of SERT Met172, would render it incapable of producing antidepressant-like effects in chronic measures of SSRI efficacy. I found that following chronic administration of vortioxetine, SERT Met172 mice were still behaviorally responsive to vortioxetine in the NIH test. Additionally, I found that the major neurogenic endpoints in the stimulation of hippocampal stem cell proliferation, as well as survival, were preserved in SERT Met172 mice. These data provide strong preclinical evidence that SERT is not required for the chronic behavioral efficacy of vortioxetine, suggesting that the residual activity of vortioxetine at serotonin receptors is sufficient to produce an antidepressant effect, both acutely and chronically.

These data indicate the potential of future serotonin directed antidepressants that completely lack activity at SERT. As much as the technological advancement occurred from MAOIs to the SERT blockers that enhance of serotonin neurotransmission, so could this next leap beyond purely antagonizing SERT to achieve serotonergic signal enhancement by engaging serotonin receptors—or other targets—directly to produce the desired quality of antidepressant pharmacotherapy. Though vortioxetine achieves this with a single molecule, it is not reasonable or necessary to do so moving forward. I perceive that future therapies in this serotonergic framework could involve a mixture of serotonin receptor specific ligands, invoking different serotonin receptors as agonists, partial agonists, or full antagonists. This receptor specificity, however, will most likely invoke allosteric mechanisms as serotonin receptors, by definition, share

high functional homology at their orthosteric binding sites, which all must bind serotonin. This approach would most likely be applied initially in the creation of serotonin receptor subtype specific allosteric modulators (both negative and positive) which could be iteratively tested alongside SSRI administration in order to achieve a higher fidelity characterization of which 5-HT receptors are necessary for the antidepressant effect of SSRIs. Once the necessary 5-HT receptors have been identified for the efficacy of SSRIs, those compounds could easily be translated to systems amenable for human preclinical trials, either singularly or combinatorial in order to produce newer more efficient serotonergic antidepressant pharmacotherapies.

This novel approach of direct selective actions at serotonin receptors would also be beneficial considering the absolute nature of SSRI pharmacotherapy, which requires nearly full saturation antagonism of the CNS systemic serotonin transporter in order to visualize any antidepressant efficacy. Depression symptom amelioration requires high SERT occupancy of SSRIs in order to achieve antidepressant effects, which produces high rates of incidence of unacceptable side effects in chronically treated individuals with depression. For the reason of side effects alone, this receptor specific approach would be very beneficial. On the other hand, with the nature of high dose necessity of SSRIs, one cannot finely control efficacy at certain 5-HT receptors, which are largely fully engaged following chronic SSRI administration, potentially leading to receptor desensitization, oversaturation, and addiction-like withdrawal sensations following pharmacotherapy cessation. It could be highly beneficial to introduce therapies that, instead of applying sledgehammer equivalent, all-or-nothing pharmacotherapies, would finely tune activity at certain serotonin receptors for subtler and better tolerated antidepressant regimens. As the field of neuroscience has accepted the CNS as more of a pathways of intercalating signaling networks, the field of pharmacology must also act accordingly and approach the depressed brain

as needing signaling recalibration instead of a whole organ that needs abrasive reshaping. It will first be necessary to achieve some receptor subtype specificity, but eventually the field of pharmacology will move to regionally-specific pharmacotherapies, though some approaches have been moderately successful in this regard, though with limited capability (Bortolozzi *et al*, 2012; Lladó-Pelfort *et al*, 2010). This regional specificity will be required as identical serotonin receptors act differently in different parts of the brain. Regional specificity will likely require elements that are not capable of single agent chemical structural elements. I anticipate that receptor specificity will be achieved with allosteric pharmacology, though region-specific delivery could likely be achieved via nanoparticle encapsulation. Nanoparticles have been studied for many years, mostly to deliver non-traditional agents past extensive first-pass metabolic mechanisms, as well as opportunities for long term delivery and extended release functionality. Recently, nanoparticles have been functionalized, allowing the penetration of a pharmaceutical compound (eg. a chemotherapeutic) and to deliver this agent preferentially to a desired site of action (eg. a tumor mass). In this example, the chemotherapeutic would normally act generally, but nanoparticle encapsulation and preferential delivery confers some regional specificity that would otherwise not be possible. This type of approach would be entirely possible given some moderate advancements in the field of nanoparticle encapsulation. In addition to the technologically capable process of developing 5-HT selective allosteric modulators, it is within grasp to create regionally specific delivery of 5-HT receptor subtype specific allosteric modulators to create a technologically superior serotonin directed pharmacological treatment compared to currently available SSRI pharmacotherapies.

APPENDICES

In addition to the experimental efforts and results presented in chapters II-V, I have also pursued other efforts relating to aspects of delineating serotonin specificity of certain drugs using the SERT Met172 mouse model. These efforts included proposed collaborations that were not ultimately pursued, or preliminary work that developed into projects for other members of the lab.

APPENDIX A. Sensitivity of the Mixed Action SNRI Antidepressant/Pain Medicines Milnacipran and Duloxetine to the SERT Met172 Substitution

APPENDIX B. Characterization of the Disrupted Pharmacological Inhibition of Cocaine upon *Ex Vivo* Derived Synaptosomes from C57Bl/6 Backcrossed SERT Met172 Mice

APPENDIX C. Adaptation of Novel Fluorescence-Based SERT Uptake Assay for Ninety-Six Well Transfected Cell Format

APPENDIX A. SENSITIVITY OF THE MIXED ACTION SNRI ANTIDEPRESSANT/PAIN
MEDICINES MILNACIPRAN AND DULOXETINE TO THE SERT MET172
SUBSTITUTION

I have described in depth in chapter II the impact of the SERT Met172 substitution upon the ability for SSRIs to inhibit SERT mediated 5-HT uptake. These analyses were performed with the intention of analyzing the 5-HT dependent and independent actions of SSRIs. The SERT Met172 model is also useful for the evaluation of multitargeted drugs that include targets at SERT, like vortioxetine in chapter V. Here, I describe the sensitivities to the multitargeted drugs, milnacipran and duloxetine, upon the SERT Met172 substitution. These drugs are newly developed mixed SERT and NET inhibitor (SNRI) that are indicated as an antidepressant as well as for fibromyalgia-type pain (Arnold *et al*, 2004; Clauw *et al*, 2008; Gendreau *et al*, 2005; Nakagawa *et al*, 2008). SNRIs are partially effective treatments for pain, due to the downwardly projecting 5-HT and NE neuronal pathways synapsing upon endogenous opioid releasing neurons, which can regulate pain signal synergy in the dorsal horn of the spinal cord (Bardin, 2011; Mochizucki, 2004; Ossipov *et al*, 2010). Here, I determine the magnitude of the degree of the disrupted affinity of milnacipran and duloxetine for SERT Met172. This aim was meant to determine whether we could disrupt the actions of these drugs at SERT, leaving the drug with only major activity at NET, in order to determine the importance of SERT actions of the drug for its efficacy in models of chronic pain.

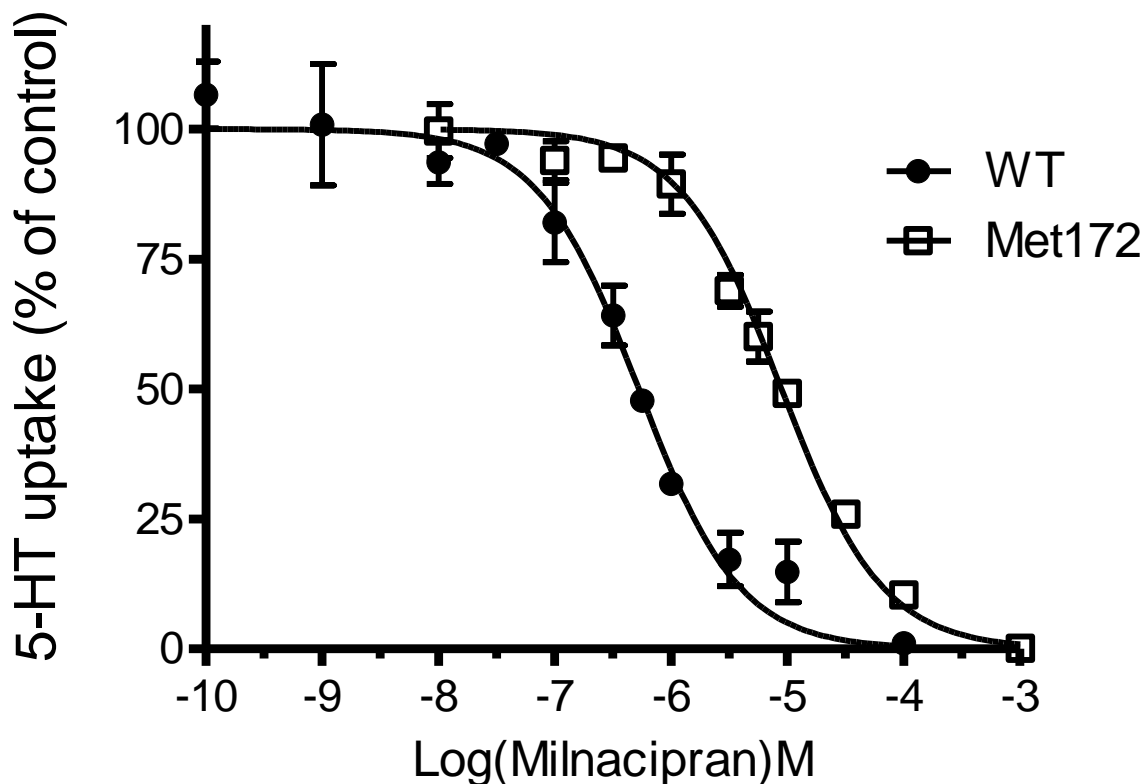
Experimental Procedure

Expanding upon the initial characterization by Julie Field in WT hSERT and SERT Met172 transfected cells, I determined whether this disrupted affinity for milnacipran and duloxetine is maintained in a more native preparation, utilizing SERT Met172 derived synaptosomes. Following sacrifice via rapid decapitation, the whole brains of 8-12 week old male WT and SERT Met172 C57Bl/6 mice were dissected on ice and used for synaptosomal preparations as described previously (Thompson *et al*, 2011). Briefly, midbrain sections were mechanically homogenized in 0.32M sucrose in 5mM HEPES buffer. This homogenate was subjected to a low gravity centrifugation (10 min, 4°C at 1,000x g) in order to remove large cellular components (eg. mitochondria, nuclei, and large membranes) from the reconstituted nerve terminals, classified as ‘synaptosomes’. The supernatant after this first spin contains the synaptosomes. The synaptosomes are then pelleted via a high gravity centrifugation (15 min, 4°C at 10,000x g). The pellet was resuspended in KRH buffer containing glucose (for synaptosomal viability throughout the preparation and assay), pargyline (MAOI, prevents enzymatic metabolism of 5-HT), and ascorbic acid (preservative, prevents spontaneous oxidation of 5-HT). Equal volumes of synaptosomes were then incubated with varying concentrations of milnacipran and duloxetine at 37°C for 10 minutes. I then added 20 nM [³H]5-HT (PerkinElmer, NET498001MC, Waltham, MA, USA) to synaptosomes and allowed 5-HT accumulation for 10 min at 37°C. 5-HT uptake into synaptosomes was terminated via addition of ice cold PBS buffer, and vacuum filtration through 0.3% polyethyleneimine treated glass fiber filters (GF/B, Whatman, Pittsburgh, PA, USA). Competition uptake inhibition curves were fit to single site competition regression curve fit (Graphpad Prism 6.0)

Results

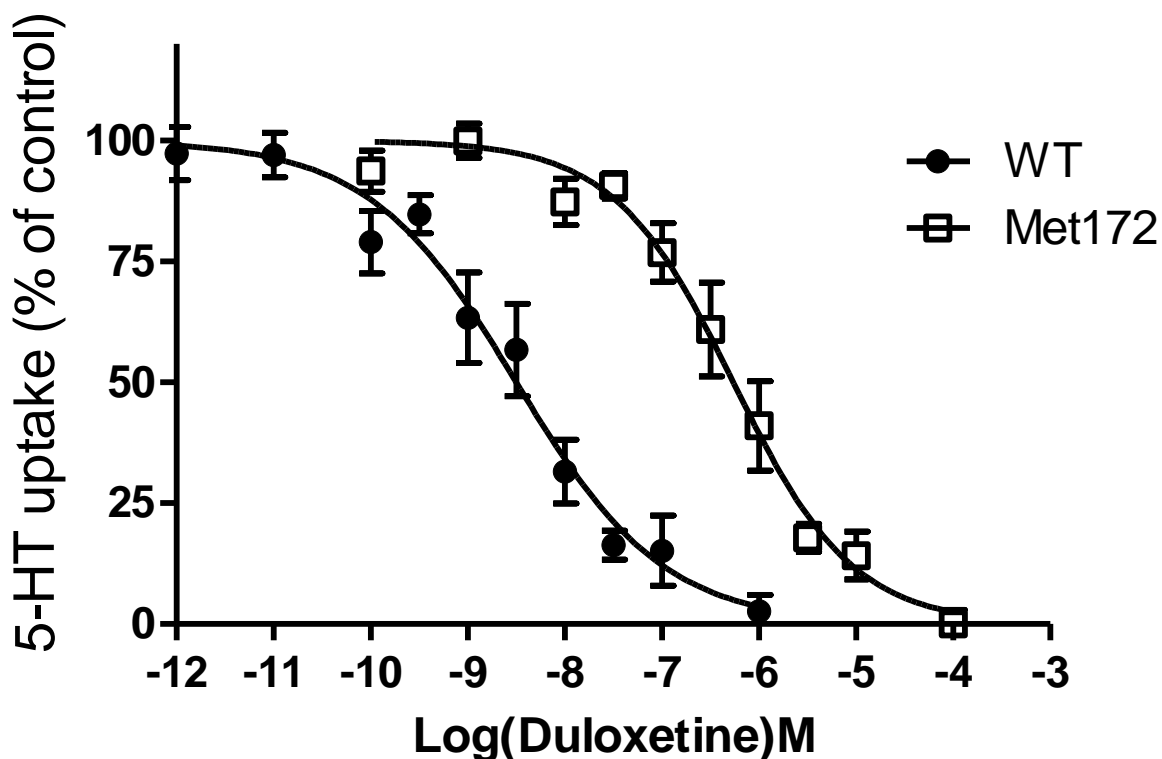
As predicted by the *in vitro* characterization performed by Julie Field, both milnacipran and duloxetine are significantly sensitive to the SERT Met172 substitution when assessed in synaptosomal preparations. Milnacipran displays a ~17-fold reduction in inhibition potency at SERT Met172 compared to WT SERT (**Appendix Figure 1**). Duloxetine displays a ~170-fold reduction in inhibition potency at SERT Met172 compared to WT SERT (**Appendix Figure 2**). Based upon these rightward shifts in inhibition efficacy for SERT Met172, and that we have demonstrated that these shifts are permissible for strategies that produce functional SERT antagonism in WT but not SERT Met172 *in vivo* (Nackenoﬀ *et al*, 2015; Thompson *et al*, 2011), we could utilize the SERT Met172 model to remove the actions of milnacipran and duloxetine at SERT to determine the importance of their actions at SERT for their efficacy as antidepressants or as analgesics.

Milnacipran 5-HT Uptake Inhibition



Appendix Figure 1. Milnacipran Sensitivity to SERT Met172 Synaptosomes. The SERT Met172 mutant imposes altered pharmacological sensitivity with normal SERT expression and function. Competition 5-HT uptake analysis in midbrain derived synaptosomes. Milnacipran (WT: K_i 526nM \pm 1.1; Met172: K_i 8.90uM \pm 1.1) was assessed for their ability to compete with [³H]5-HT uptake. (n = 4/condition). Like the *in vitro* studies, milnacipran displays reduced potency for SERT Met172 vs WT SERT (~17-fold reduction).

Duloxetine 5-HT Uptake Inhibition



Appendix Figure 2. Duloxetine Sensitivity to SERT Met172 Synaptosomes. The SERT Met172 mutant imposes altered pharmacological sensitivity with normal SERT expression and function. Competition 5-HT uptake analysis in midbrain derived synaptosomes. Duloxetine (WT: K_I 17.1nM \pm 2.0; Met172: K_I 262nM \pm 2.0) was assessed for their ability to compete with [3 H]5-HT uptake. (n = 4/condition). Like the *in vitro* studies, duloxetine displays reduced potency for SERT Met172 vs WT SERT (~170 fold reduction).

APPENDIX B. CHARACTERIZATION OF THE DISRUPTED PHARMACOLOGICAL
INHIBITION OF COCAINE UPON *EX VIVO* DERIVED SYNAPTOSOMES FROM C57BL/6
BACKCROSSED SERT MET172 MICE

I have described earlier, in chapter II, the impact of the SERT Met172 substitution upon the ability for SSRIs to inhibit SERT mediated 5-HT uptake. These analyses were performed with the intention of analyzing the 5-HT dependent and independent actions of SSRIs. The SERT Met172 model is also useful for the determination of the importance of SERT antagonism for multitargeted drugs, that include SERT among their targets. Cocaine produces strong neuropsychotropic effects via its actions at DAT, SERT, and NET, where it functions as a reuptake blocker at all three of those proteins upon their respective neurotransmitter (Uhl *et al*, 2002). We have shown in lab previously that cocaine is sensitive to the SERT Met172 substitution (Henry *et al*, 2006; Thompson *et al*, 2011). Here, I expand upon those efforts and test whether this disrupted affinity of cocaine at SERT Met172 is maintained a more relevant reconstituted system for further assessment of *in vivo* SERT-dependent actions of cocaine.

Experimental Procedure

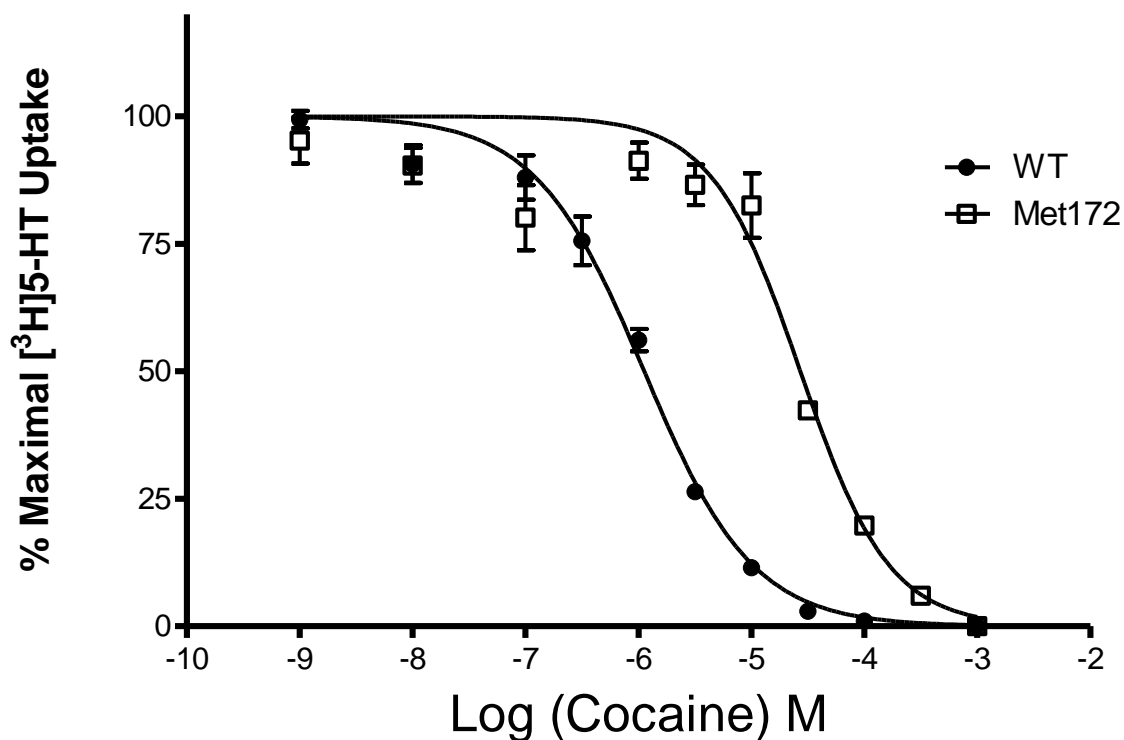
Expanding upon the initial characterization by Keith Henry, Brent Thompson, and Julie Field in WT hSERT and SERT Met172 transfected cells and synaptosomes of 129S6/S4 SERT Met172 mice, I determined whether this disrupted affinity for cocaine is maintained in a more native preparation, utilizing SERT Met172 derived synaptosomes. Following sacrifice via rapid decapitation, the whole brains of 8-12 week old male WT and SERT Met172 C57Bl/6 mice were

dissected on ice and used for synaptosomal preparations as described previously (Thompson *et al*, 2011). Briefly, midbrain sections were mechanically homogenized in 0.32M sucrose in 5mM HEPES buffer. This homogenate was subjected to a low gravity centrifugation (10 min, 4°C at 1,000x g) in order to remove large cellular components (eg. mitochondria, nuclei, and large membranes) from the reconstituted nerve terminals, classified as ‘synaptosomes’. The supernatant after this first spin contains the synaptosomes. The synaptosomes are then pelleted via a high gravity centrifugation (15 min, 4°C at 10,000x g). The pellet was resuspended in KRH buffer containing glucose (for synaptosomal viability throughout the preparation and assay), pargyline (MAOI, prevents enzymatic metabolism of 5-HT), and ascorbic acid (preservative, prevents spontaneous oxidation of 5-HT). Equal volumes of synaptosomes were then incubated with varying concentrations of cocaine at 37°C for 10 minutes. I then added 20 nM [³H]5-HT (PerkinElmer, NET498001MC, Waltham, MA, USA) to synaptosomes and allowed 5-HT accumulation for 10 min at 37°C. 5-HT uptake into synaptosomes was terminated via addition of ice cold PBS buffer, and vacuum filtration through 0.3% polyethyleneimine treated glass fiber filters (GF/B, Whatman, Pittsburgh, PA, USA). Competition uptake inhibition curves were fit to single site competition regression curve fit (Graphpad Prism 6.0)

Results

As predicted based upon earlier characterizations, cocaine displays disrupted ability to inhibit SERT mediated 5-HT uptake in SERT Met172 derived synaptosomes, rendering a ~25-fold rightward shift in inhibition potency (**Appendix Figure 3**).

Cocaine 5-HT Uptake Inhibition



Appendix Figure 3. Cocaine Sensitivity to SERT Met172 Synaptosomes. The SERT Met172 mutant imposes altered pharmacological sensitivity with normal SERT expression and function. Competition 5-HT uptake analysis in midbrain derived synaptosomes. Cocaine (WT: K_I 1.12 μ M \pm 1.1; Met172: K_I 27.1 μ M \pm 1.1) was assessed for their ability to compete with [³H]5-HT uptake. (n = 4/condition). Like the *in vitro* studies, cocaine displays reduced potency for SERT Met172 vs WT SERT (~25-fold reduction).

Based upon previous efforts to select doses of SSRI that provide functional antagonism in SERT but not SERT Met172 *in vivo* (Nackenoff *et al*, 2015; Thompson *et al*, 2011), it should be possible to achieve a similar result with cocaine in the SERT Met172 model. This would allow the selective removal of SERT antagonism from the pharmacological properties of cocaine, and any residual effects of cocaine would not be dependent upon its actions at SERT, and conversely any effects lost would be due to the actions of cocaine at SERT.

I oversaw the efforts of initial characterizations of SERT dependent behavioral effects of cocaine in the SERT Met172 model, an undergraduate honors project of Peter Chisnell upon the 129S6/S4 genetic background SERT Met172 mice. Dr. Linda Simmler inherited this project in our lab following the backcrossing of the SERT Met172 mice onto the C57Bl/6 background. My findings also initiated collaborations that investigated the dependency of cocaine actions at SERT to trigger cocaine induced alterations in circadian rhythm. These efforts found that the well-described actions of cocaine to alter circadian rhythm is in fact due to its actions at SERT, as cocaine-induced alterations in circadian rhythm are lost in SERT Met172 mice (Prosser *et al*, 2014).

Our main focus in the lab, however, has been to investigate the role of serotonin to modulate or support cocaine addiction related behaviors. In order to specifically analyze the specific roles of serotonin in cocaine action, we will need to pursue more complicated contingent measures of cocaine addiction. We have traditionally utilized locomotor chambers to analyze cocaine induced increases in locomotion, which is thought to measure raw cocaine sensitivity, but because cocaine is administered non-contingently, cannot measure desire to consume or pleasure derived from cocaine. Linda Simmler engaged in conditioned place preference (CPP) assays, which aim to measure how much the animal attains pleasure from consuming cocaine, though this

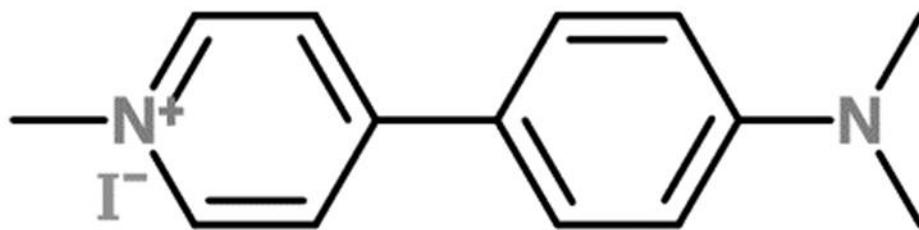
measure cannot measure drive to consume as cocaine is administered non-contingently. In order to assess the subtler modifications to addiction related behaviors, in which serotonin could be modulating, we need to investigate whether removal of SERT antagonism from cocaine action modulates contingent drug seeking behavior. To accomplish this, we need to initiate cocaine self-administration paradigms. Optimally, this would entail cranial intraventricular injected cocaine upon an operant trained behavior (eg. nose poke), though this would require complicated individual surgeries upon all mice utilized for the study. Alternatively, one could administer cocaine through small operant behavior-mediated deliveries of cocaine infused water, though the route of administration is not as prompt as intracranial delivery, this would alleviate the burden of large number of stereotaxic surgeries required for intracranial cocaine delivery. Regardless of the method, the self-administration paradigm allows for the investigation into how desirable animals view cocaine, because the animal is able to determine the speed and total amount of cocaine deliveries over a session. Once basic parameters are established, one could modulate the operant paradigm to test the limits of drug seeking behavior, the strongest measure of which would be to deliver cocaine upon a progressive ratio, which increases the requirements placed upon the animal to access the next delivery of cocaine (eg. progressively increasing the number of nose pokes required for next subsequent delivery of cocaine). This tests the ‘break point’, which is a measure into the maximal amount of effort that an animal will expend in order to acquire the next delivery of cocaine.

Another important measure would be to investigate alterations in withdrawal/extinction following self-administration. Additionally, we should also assess the role of serotonin on cocaine action for stress or cue induced reinstatement of drug seeking behavior. Should the actions of cocaine on serotonin modify the drug seeking behavior, SERT Met172 mice would display

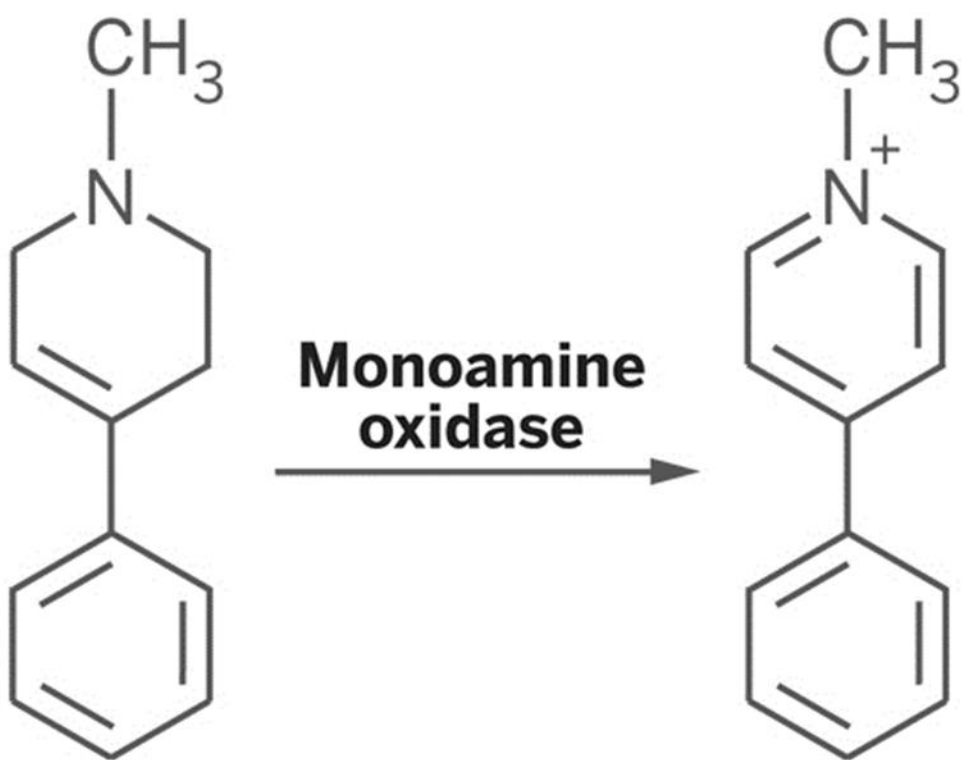
alterations in their ‘break point’ and/or reinstatement behavior compared to WT mice, which would illustrate how serotonin modulates cocaine actions, and potentially illuminating new serotonergic mechanisms to target for future pharmacotherapies alleviating cocaine addiction.

APPENDIX C. ADAPTATION OF NOVEL FLUORESCENCE-BASED SERT UPTAKE
ASSAY FOR NINETY-SIX WELL TRANSFECTED CELL FORMAT

All previously described SERT specific uptake assays in the preceding document utilize radiolabeled [³H]5-HT. The use of radiolabeling presents numerous problems. Firstly, radiolabeling is a safety hazard, necessitating proper personal protection, storage, and disposal to ensure minimal human and environmental contact to sources of ionizing and non-ionizing radiation. Additionally, while the use of radiolabeling provides a source of direct measurement of uptake, it can only do so as an endpoint analysis, where potentially critical information of the nature of uptake is lost and unable to be captured. In order to address these highlighted concerns, a fluorescent substrate was developed for the monoamine systems DAT, NET, and SERT (Blakely *et al*, 2011). This compound, IDT307, (**Appendix Figure 4**), is a functional derivative of MPP⁺ (an active metabolite of the infamous MPTP) which itself is a fluorescent substrate of DAT and NET, though IDT307 possesses much increased substrate capacity for SERT, allowing it to serve as a general fluorescent substrate for all three transporter systems (Blakely *et al*, 2011). Importantly, this fluorescence only occurs when the compound is internalized, allowing for high signal to noise and real time administration of uptake. Here, I describe my efforts to adapt and create efficient cell transfection for use in 96-well format for higher throughput analyses of modulations of SERT uptake.



IDT307



MPTP

MPP⁺

Appendix Figure 4. Structure of IDT307. Comparison of the structure of IDT307 to MPTP and its metabolite MPP⁺

Experimental Procedure

In order to prepare the cells for efficient transfection, I first setup the transfection in tube format, add suspended cells, and then plate this cell and transfection mix to 96-well plates for further experimentation. I explain this now in detail.

In 14 mL polystyrene tubes (Falcon), I add 2.5 mL of OptiMEM, and then add TransIT (Mirus) (at a ratio of 3uL TransIT per 1ug DNA transfected). I then swirl to mix, and let incubate at room temperature for 10 minutes, after which I add the appropriate amount of DNA and mix gently, followed by a 15-minute incubation. Here, I perform 5ug transfections of hSERT containing pcDNA3 plasmids (Invitrogen) (assuming a full 96 well plate transfection). I then take cultured cells and suspend them following a trypsin digest and resuspension in cell media. HEK-293T cells were cultured at 37°C in modified DMEM cell media containing 10% dialyzed fetal bovine serum (to eliminate 5-HT in growth media) and antibiotics. Once cells are suspended in new culture media, I count the cell concentration on a hemocytometer. From this, I calculate the volume needed for 5×10^6 cells and add this to the OptiMEM transfection reagent tube and wait 20 minutes. After this, I adjust the volume of the reaction tube to 10 mL, adding fresh cell media. This cell transfection suspension is then dispensed at 100 uL per well to poly-D-lysine coated (to aid adherence) 96 well black sided clear-bottomed plates, and cells allowed to grow at 37°C for at least 24 hours (best results are found with 36 hours).

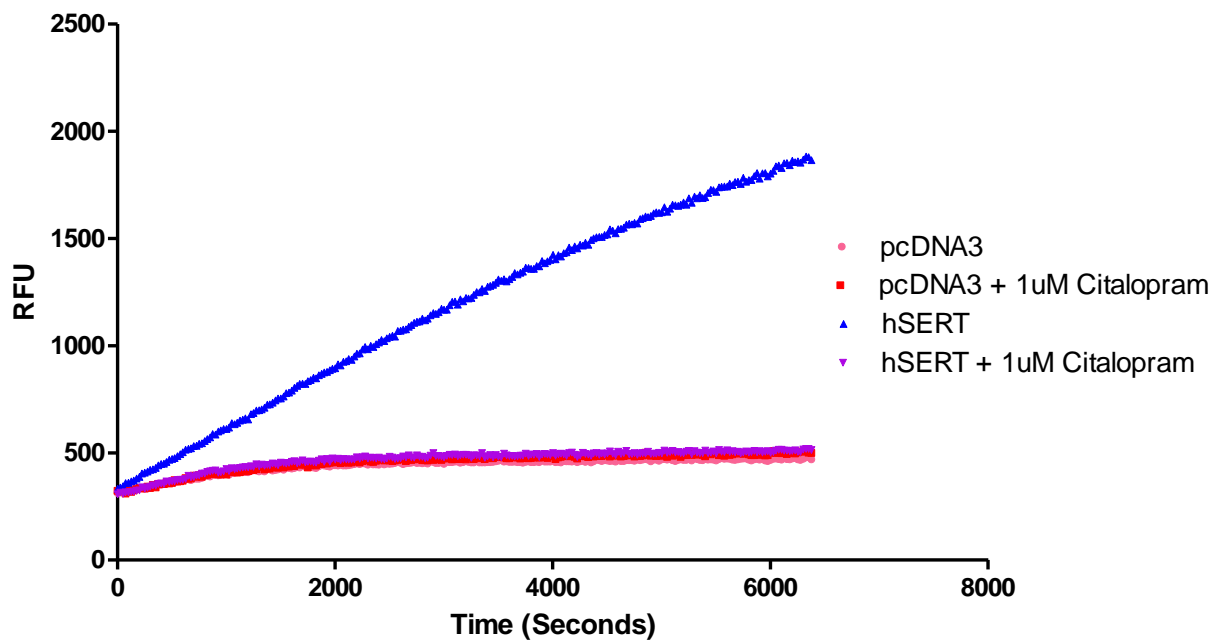
On test day, cell media was aspirated off of the cells and replaced with Krebs-Ringer HEPES buffer (KRH) assay buffer containing pargyline (MAOI, prevents enzymatic metabolism of 5-HT), and ascorbic acid (preservative; prevents spontaneous oxidation of 5-HT). Cells were then preincubated with drugs for 10 minutes at 37°C. Cells were then incubated with 1 uM (final)

IDT307 and allowed to accumulate at 37°C. Immediately following administration of IDT307 to the wells, the plate is placed into the fluorescence measurement chamber (Flexstation) and assessed for uptake into cells. Excitation laser was set at 440nm and emission detection was set at 520nm.

Result

The purpose of this effort was to establish an easier and more reliable protocol for 96-well fluorescent IDT307 uptake assays in transiently transfected cells. These efforts mainly focused upon the transfection procedure. Traditional means of transfecting cells in 12 or 24 well format are easily suited for individual transfection of each well of cells (previously grown in a monolayer), though the 96 well format presents a problem for reliably doing so. Small variations in transfection reagent adherence to pipet tips of small volume administrations present potential large variability in transfection efficiency, causing inter-well variability independent of any treatment modulation. By transfecting and plating cells at the same time, this reduces the variability induced by individual well transfection procedures.

Here, I demonstrate the efficacy of the procedure. As shown, clear SERT-specific IDT307 accumulation is seen in cells when compared against empty vector transfections and citalopram (SSRI) inhibited uptake (**Appendix Figure 5**). This assay can be used for situations that require higher throughput of transfection of multiple SERT variants or drug screening procedures which would not be best suited utilizing inefficient smaller plate formats. Though, an important caveat should be illuminated to any findings generated using IDT307 as opposed to 5-HT uptake studies.



Appendix Figure 5. Example trace of SERT-specific IDT307 accumulation. Fluorescence accumulation was conducted in the presence of 1uM IDT307 in HEK-293T cells transfected with either empty vector (pcDNA3) or hSERT, either uninhibited or in the presence of the SSRI citalopram (1uM).

As IDT307 and 5-HT are structurally dissimilar, there may be substrate-specific mechanisms that support/disrupt the actions of one substrate that may not be consequential for the other. Therefore, it may be useful to utilize IDT307 uptake assays when the situation dictates the screening of a large number of modulations, though important findings with IDT307 should ultimately be confirmed upon traditional substrate systems with smaller [³H]5-HT based uptake assays.

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