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

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

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B.S., Lafayette College, 2008

Advisor: Michael J. Owens, PhD

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Abstract

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By Karen Elaine Murray

Escitalopram is a commonly prescribed antidepressant of the selective serotonin reuptake inhibitor class. Clinical evidence followed by pre-clinical evidence and mapping on the serotonin transporter (SERT) identified that in addition to inhibiting the SERT via a primary site, escitalopram is capable of binding to the SERT via an allosteric site. *In vitro* studies suggest that the allosteric site alters the kinetics of escitalopram at the SERT. This dissertation examined the *in vivo* role of the allosteric site in escitalopram action at the SERT. This was completed by developing a knockin mouse model that had an allosteric-null SERT. Autoradiographic studies indicated that the knockin protein was expressed at a lower amount than endogenous mouse SERT, but the knockin mice were a viable tool to study the allosteric site. It was hypothesized that the absence of the allosteric site would result in the need for a higher dose of escitalopram to achieve the same effect seen in mice with intact SERT. Microdialysis studies in the ventral hippocampus found no measurable decrease in the amount of extracellular serotonin after escitalopram challenge in mice without the allosteric site. In marble burying assays there was a modest effect of the absence of the allosteric site, with a larger dose of escitalopram necessary to see the same effect as in mice with intact SERT. In the tail suspension test there was no effect of the presence or absence of the allosteric site. Together these data suggest that there may be a regional specificity in the role of the allosteric site, explaining the modest marble burying effect without matching tail suspension test and microdialysis effects. The knockin mice could be used to explore this further. However, the lack of a robust effect overall suggests that the role of the allosteric site for escitalopram on the SERT does not produce relevant *in vivo* effects.

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Chapter 1: Introduction and Background

Depression and Treatments

Psychiatric illness affects millions of Americans each year. Major depressive disorder affects many patients and is frequently comorbid with a range of other diseases and disorders including but not limited to anxiety disorders, obsessive-compulsive disorder, posttraumatic stress disorder, Alzheimer's Disease, and stroke (Chapman, Perry, & Strine, 2005; Katon, Lin, & Kroenke, 2007; Strine et al., 2008). Consequently, treatment of depression is a billion dollar industry with great interest in developing better treatment for patients (Nisen, 2012). Treatments include behavioral therapies, such as cognitive behavioral therapy, somatic therapies, such as electroconvulsive therapy, and pharmaceutical interventions, such as Prozac (Blier & de Montigny, 1994; National Institute of Mental Health: Depression Booklet, 2011). Unfortunately for depressed patients there is no singularly and completely effective course of treatment to permanently eradicate their depression. Some patients never obtain remission of their depressive symptoms, and many of those who do respond go on to relapse or never achieve full remission (Blier, 2013; Nierenberg et al., 2010). Additionally, one course of treatment may be effective in one patient but have minimal to no effect in another patient who otherwise appears similar (Healy, 2000; Möller, 2000). While the differential patient responses to individual treatments may speak to the idiopathic nature of this neuropsychiatric disease, in depth examination of the mechanism of action of a treatment may reveal a greater understanding of not only the treatment itself but depression pathophysiology. This could then lay the groundwork for future treatment development that may be more comprehensive and effective.

Pharmaceutical interventions are an attractive treatment avenue for study. Laboratory animal models and procedures can be developed to break down and individually study each step of a compound's mechanism of action. A molecule by molecule building to a cellular and systems level approach is not as accessible for study in treatments other than pharmaceutical interventions. Additionally, using compounds that are known to target a specific molecule or receptor can be used to implicate that molecule or target in the pathology of depression. Historically, monoaminergic pathways in the brain have been implicated in depression because compounds that modulate monoamines have been effective treatments (Blier & de Montigny, 1994; Blier, 2013; Booij, Van der Does, & Riedel, 2003; Fuller & Wong, 1990; Fuller, 1995; Hirschfeld, 2000; Ressler & Nemeroff, 2000). Another example includes corticotropin-releasing factor, for which an antagonist to that pathway demonstrated relief of symptoms in some patients (Bourke & Owens, 2010). Non-pharmaceutical therapies may result in changes to these implicated systems, but manipulations by pharmaceutical agents provide a definitive tool.

Of the pharmaceutical compounds designed to treat depression, selective serotonin reuptake inhibitors (SSRIs) are the drug class most frequently prescribed to depressed patients. SSRIs act by disrupting the serotonin transporter's (SERT) movement of serotonin into cells (Fuller & Wong, 1990; Fuller, 1995). This results in accumulation of serotonin outside of the cells which leads to increased activation of serotonin receptors. Due to the known ability of SSRIs to alleviate depressive symptoms, it is hypothesized that the accumulation of extracellular serotonin results in prolonged serotonergic activity that has downstream effects that reduce depressive symptoms

(Faure, Mnie-Filali, & Haddjeri, 2006; Owens & Nemeroff, 1994; Ressler & Nemeroff, 2000). The downstream pathways are not fully elucidated, although it is known that the serotonergic system does not act in isolation to achieve symptom reduction because serotonin acts on other systems and manipulation of other targets can cause symptom relief (Blier & de Montigny, 1999; Blier, 2013; Owens, 1997).

Despite not having a full downstream mechanism of action described, inhibiting the SERT via SSRIs has been a key focus of pharmaceutical treatment of depression. Numerous SSRIs were developed in the twentieth century, with several becoming blockbuster compounds and even household names, and they have become the first-line treatment option for depression. These commonly prescribed SSRIs include fluoxetine, sertraline, paroxetine and citalopram, also known by their trade names of Prozac, Zoloft, Paxil, and Celexa, respectively. As a drug class, SSRIs all have the same mechanism of action: inhibit the SERT. Most SSRIs bind to the same region of the SERT, although different individual amino acids may be implicated for singular compounds (Andersen et al., 2010; Mortensen, Kristensen, & Wiborg, 2001; Roman, Walline, Rodriguez, & Barker, 2003; Tavoulari, Forrest, & Rudnick, 2009). Although no crystal structure of the SERT is available for any species, the SERT's homology to the bacterial leucine transporter and drosophila dopamine transporter suggest that SSRIs bind around the SERT's central pocket for transporting serotonin (Penmatsa, Wang, & Gouaux, 2013; Singh, Yamashita, & Gouaux, 2007). The variety in the SSRI class is generally due to differences between the compounds in selectivity, sensitivity, and pharmacokinetics. Some compounds are not selective and have numerous off target interactions (Owens, Morgan, Plott, & Nemeroff, 1997); some compounds actually show so much additional

activity at the norepinephrine transporter (NET) that they are instead classified as serotonin-norepinephrine reuptake inhibitors, such as venlafaxine and duloxetine. Some compounds are not very sensitive in their interaction with the SERT and have a low affinity for the SERT, thus a high concentration is required to achieve any activity. Ideal SSRIs will have high selectivity and sensitivity. The assumption that follows is that such compounds will only target the serotonergic system and patients will be able to use a low dose of the compound to further prevent any non-specific or off-target interactions that inevitably occur at high drug concentrations.

In the clinic, SSRIs are the first line prescription for depression, and often the first line treatment plan for new patients. Unfortunately SSRIs are not uniformly successful. For unknown reasons, SSRIs will fail entirely in some patients. Relief is achieved in many patients, though it typically takes several weeks from the beginning of treatment until improvement begins (Blier & de Montigny, 1994; Gelenberg & Chesen, 2000; Owens & Nemeroff, 1994). This lag is likely due to changes in the serotonergic and downstream systems after beginning SSRI treatment, with adaptive changes in serotonin autoreceptors noted as key (Diaz et al., 2011; Getz, Xu, Zaidi, & Syed, 2011; Ichimaru, Egawa, & Sawa, 1995; Santarelli et al., 2003). In the treatment of depression, SSRIs are mainly meant to act on the neural serotonergic system, but SSRIs are typically delivered systemically in tablet form, and SSRIs are thus able to act on the SERT outside of the brain, resulting in numerous side effects via serotonergic systems outside the brain (Berger, Gray, & Roth, 2009; Fuller & Wong, 1990). The lag in treatment effect along with potential side effects makes patient compliance an issue. It can be tough for a patient who is hoping to get better quickly to have to wait for the changes to the serotonergic

system for eventual symptom relief. Additionally, because much of the serotonin in the human body is located outside the nervous system and thus SSRIs will act there, this results in acute effects that are undesirable for patients, including gastrointestinal, sexual and metabolic effects. SSRI development has focused on selectivity and potency in an effort to remedy these issues, particularly the side effects.

Citalopram is a very selective and sensitive SSRI (Owens, Knight, & Nemeroff, 2001; Owens & Rosenbaum, 2002). Its affinity for the SERT is in the low nanomolar range and it has no physiologically relevant interactions besides the SERT. While it is not wholly immune from the limitations of other SSRIs, citalopram is an effective antidepressant (Hyttel, 1982). Citalopram is a racemic compound, the *S*-enantiomer, escitalopram, is the racemate with the biologically relevant SSRI activity (Hyttel, Bøgesø, Perregaard, & Sánchez, 1992). While *R*-citalopram is able to inhibit the SERT, escitalopram's affinity is 30-fold greater than *R*-citalopram's, and *R*-citalopram is not an effective SSRI at physiologically relevant doses (Owens et al., 2001). Due to being the principal active enantiomer, escitalopram was developed into a successful stand alone, enantiopure drug.

Although citalopram was previously approved to treat depression in the United States and Europe, escitalopram had to be approved separately. This was expected to be a formality given that citalopram treatment is essentially escitalopram treatment with the expectation that *R*-citalopram has no effect. Studies in depressed patient populations compared placebo and citalopram to escitalopram (Auquier, Robitail, Llorca, & Rive, 2003; Gorman, Korotzer, & Su, 2002; Lepola, Loft, & Reines, 2003; Lepola, Wade, & Andersen, 2004). Citalopram and escitalopram were administered in equimolar doses so

that the patients in the drug arms all received the same amount of escitalopram, the only difference being the citalopram arm patients received seemingly inert *R*-citalopram in addition. The intention of the trials was to indicate that escitalopram was non-inferior compared to citalopram. Surprisingly, early trials indicated that escitalopram was actually superior to citalopram. Patients in escitalopram arms achieved improvement faster and more frequently than patients in the citalopram or placebo arms (Lepola et al., 2003, 2004). This improvement is the goal of SSRI development, so studies moved to the laboratory from the clinic in order to better understand escitalopram's mechanism of action with the ultimate goal to achieve a greater understanding of SSRIs action in depression treatment to develop more effective future treatments.

Allosterism

Initial hypotheses about the variation in escitalopram's activity compared to the racemic citalopram included an allosteric site for escitalopram on the SERT. Allosterism was an attractive solution because allosteric activity would explain how escitalopram could be having a greater effect without implicating a pathway of activity that did not include the SERT. Citalopram was known to be very selective for the SERT, a characteristic attributed to the *S*-enantiomer, thus it appeared unlikely that removing the *R*-enantiomer would suddenly allow for the *S*-enantiomer to act on a non-SERT pathway. Allosteric activity would indicate that escitalopram was acting in a second location on the SERT, affecting action at the primary location (Kenakin, 1997). While escitalopram acting as its own allosteric compound is unusual, allosterism was a logical explanation for the clinical findings and mapping a second location for escitalopram binding on the human transporter (hSERT) bolstered this theory (Neubauer, Hansen, & Wiborg, 2006).

Additionally, allosterism is a common mechanism by which compounds augment or moderate activity that occurs in a primary binding site. Escitalopram's putative allosteric activity will be further discussed in the next section, but first a discussion of allosterism in general.

Allosterism is frequently found as a mechanism by which compounds modulate the activity of other compounds. First described in the 1960s in work by Changeux, and developed into the Monod-Wyman-Changeux (MWC) model, allosteric activity was identified as a type of cooperativity by two effector molecules on an enzyme or receptor molecule (Changeux, 2013; Kenakin, 1997; Monod, Changeux, & Jacob, 1963; Monod, Wyman, & Changeux, 1965). Hemoglobin was one of the first molecules identified to have allosteric activity. As oxygen molecules bind to the heme group they alter the larger molecule, transitioning the hemoglobin molecule from the oxygen-free T state to the oxygen-bound R state and altering hemoglobin's affinity for oxygen (Motlagh, Wrabl, Li, & Hilser, 2014; Perutz, Wilkinson, Paoli, & Dodson, 1998). Effectively the first bound oxygen modulates the hemoglobin to allow for the next bound oxygen (Kenakin, 1997). Overall this action is separate from the orthosteric, or primary, ligand interaction on the receptor. The key to allosteric ligands is that they do not affect the receptor action or active site directly; rather they alter the relationship of the orthosteric ligand and the receptor to modulate the response of that action.

As the understanding of allosterism grew, the MWC model of allosteric activity was supported and identified in numerous ligand-receptor pairs. Notably allosteric activity was discovered to be a mechanism of action for several neurally active compounds: benzodiazepines, barbiturates, galantamine, and sertraline.

Benzodiazepines are a drug class that act on the $GABA_A$ receptor, and are widely prescribed as sleep medications (Gao & Jacobson, 2006). They act by increasing the probability that the $GABA_A$ chloride channel will open in the presence of GABA (Gao & Jacobson, 2006; Macdonald & Olsen, 1994; Smith & Olsen, 1995). The key is that benzodiazepines do not open the channel directly, rather increase opening probability when GABA is bound to the ionotropic receptor (Macdonald & Olsen, 1994; Smith & Olsen, 1995). GABA acts endogenously at the $GABA_A$ receptor, with or without benzodiazepines present. Additionally, the binding sites of GABA and benzodiazepines are distinct and unlike direct competitors of GABA binding, allosterically acting benzodiazepines have a better side effect profile to make them more tolerable drugs for use compared to direct $GABA_A$ agonists (Smith & Olsen, 1995). This mechanism is similar to how two escitalopram molecules are able to bind to the SERT in two distinct locations (described later); however the role of the allosterically acting escitalopram molecule is not as well-defined as the role of benzodiazepines on the $GABA_A$ receptor.

Also acting at the $GABA_A$ receptor, barbiturates enhance the affinity of GABA binding. Barbiturates are used as sedatives, anesthetics, and anticonvulsants, and do possess some action on the receptor when GABA is absent (Macdonald & Olsen, 1994). Binding in a location distinct from GABA and benzodiazepines, barbiturates are able to modulate GABA's actions on the $GABA_A$ receptor by increasing the channel's time open duration (Macdonald & Olsen, 1994). Similar to escitalopram, barbiturates have some primary activity, but additionally they are also able to enhance the primary ligand's actions. Although in the case of escitalopram those ligands are one and the same.

The nicotinic acetylcholine receptor (nAChR) is also a locus of allosteric activity, specifically for the Alzheimer's Disease medication galantamine. Similarly to the $GABA_A$ receptor, directly acting compounds at nAChRs have proven to be less than desirable in the clinic, ultimately achieving a loss of activity (Villarroya, García, Marco-Contelles, & López, 2007). Galantamine is considered an allosteric potentiating ligand of the nAChR, and it is suggested that the positive effects the compound has on Alzheimer's Disease are due to its ability to enhance the excitatory function of the nAChRs (Villarroya et al., 2007).

Allosteric activity has also been suggested for SSRIs besides escitalopram. Sertraline slows the dissociation of nisoxetine from the NET (Plenge & Mellerup, 1997). This is even though sertraline's affinity for the NET is much less than its affinity for the SERT (Owens et al., 1997). This pattern of sertraline allosteric activity at the NET is similar to a pattern observed for escitalopram at the SERT (Plenge & Mellerup, 1997). It is important to note that sertraline is considered to be of the SSRI class and its principle clinical action is considered to be at the SERT, but the observed effect at the NET could be a supplemental action to the antidepressant effect as norepinephrine is also hypothesized to play a role in depression etiology and treatment. Currently this sertraline allosteric activity at the NET is not being actively pursued as a valuable mechanism for pharmacotherapy.

While the aforementioned allosteric effector compounds are affecting different compound from themselves, the initial hypotheses for escitalopram's allosteric activity suggested that another escitalopram molecule acting on the SERT modulated the primary, or orthosteric, escitalopram molecule's activity at the SERT. This is somewhat in line

with the actions of oxygen at hemoglobin, with oxygen molecules increasing the affinity of the hemoglobin for oxygen, allowing for the next molecule to bind. Escitalopram in the allosteric site on the SERT is not hypothesized to alter the affinity of primary escitalopram binding, and primary affinity has been observed to be stable in the absence of the allosteric site (Neubauer et al., 2006). Instead, escitalopram seems to alter the on- and off-rate kinetics by keeping escitalopram bound to the SERT for a longer period of time (Chen, Larsen, Neubauer, et al., 2005; Plenge & Mellerup, 1997). The putative allosteric site for escitalopram will be discussed fully in the next section.

Allosteric Site

The surprising clinical differences between escitalopram and citalopram led to a return to the laboratory from the clinic in order to identify the underlying cause of the difference. The phenomenon was replicated in rodent models and screens of SSRI activity. Microdialysis studies in rodents found that escitalopram challenge increases extracellular concentrations of serotonin, as would be expected by an SSRI (Mørk, Kreilgård, & Sánchez, 2003; Nguyen et al., 2013; Thrivikraman, Kinkead, Murray, & Owens, 2013). Further, *R*-citalopram alone does not elicit such an increase, but the addition of *R*-citalopram to escitalopram challenge blunts the extracellular serotonin increase seen with escitalopram alone (Mørk et al., 2003). *R*-citalopram also blunts the inhibition by escitalopram on dorsal raphe nucleus neuron firing (El Mansari, Sánchez, Chouvet, Renaud, & Haddjeri, 2005). Escitalopram decreases spike amplitude of CA1 hippocampal neurons, but *R*-citalopram blunts this effect (Mnie-Filali, El Mansari, Espana, Sánchez, & Haddjeri, 2006). Many behavioral assays in rodents also mimic this effect of *R*-citalopram blunting the effect of escitalopram (Sánchez, Gruca, Bien, & Papp,

2003; Sánchez, Gruca, & Papp, 2003; Sánchez & Kreilgård, 2004; Sánchez, 2003; Sánchez, Bergqvist, et al., 2003; Stórustovu et al., 2004). The replication in animal models of the clinical data that escitalopram has greater effects alone than as a racemic mixture laid the groundwork for further investigation of what was causing this phenomenon. What follows is the history of evidence for the allosteric site.

Studying the SERT *in vitro* found that escitalopram at moderate concentrations retards dissociation rates of escitalopram-SERT complexes (Chen, Larsen, Neubauer, et al., 2005; Plenge, Gether, & Rasmussen, 2007). Escitalopram is also able to slow the dissociation rates of other SSRIs bound to the SERT, including imipramine, paroxetine, fluoxetine, sertraline, and venlafaxine (Chen, Larsen, Sánchez, & Wiborg, 2005). *R*-citalopram, sertraline, paroxetine and serotonin also have some level of slowing dissociation of SSRIs from the SERT, but none to the extent of escitalopram (Chen, Larsen, Sánchez, et al., 2005). Other SSRIs, including fluoxetine and fluvoxamine, do not show this activity (Chen, Larsen, Sánchez, et al., 2005; Mortensen et al., 2001). Additionally, the association rate of citalopram is disrupted by increasing concentrations of *R*-citalopram, further suggesting an allosteric site as a mechanism to alter escitalopram's kinetics (El Mansari et al., 2007).

Work in rodents focused on characterizing escitalopram's physiological and behavioral activity. There has also been a focus comparing escitalopram with racemic citalopram, to mimic the clinical effects. The differences between the enantiopure and racemic compound in humans were replicated in mouse and rat models: animals have a faster response in the escitalopram groups compared to citalopram. This has been seen physiologically in rats where microdialysis in the frontal cortex revealed subcutaneous

injection of escitalopram resulted in increased extracellular serotonin, while the addition of increasing doses of *R*-citalopram blunts the serotonin response (Mørk et al., 2003). It is important to note that in that particular study *R*-citalopram alone, at the utilized doses, did not evoke an increase in extracellular serotonin. The effects of *R*-citalopram were only revealed in conjunction with escitalopram. And because the affinities of the two enantiomers for the hSERT are so different, suggesting *R*-citalopram cannot out-compete escitalopram at the primary locus for inhibiting the hSERT, this data suggests a secondary or allosteric locus of action where the two molecules may compete (Owens et al., 2001). Behavioral results, including in ultrasonic vocalization, elevated plus maze, and Vogel conflict, have also matched the findings of the microdialysis, that the addition of *R*-citalopram reduces the escitalopram effect (Sánchez, 2006; Sánchez, Bergqvist, et al., 2003).

Together, the rodent microdialysis and behavior tests with the *in vitro* kinetic studies suggested the presence of an allosteric site for escitalopram. Species-scanning mutagenesis studies confirmed and mapped the allosteric site. Although the SERT has a high degree of homology across species, there are some variations in the SERT species to species. The *in vitro* dissociation studies were performed using the human SERT (hSERT). The chicken SERT (gSERT) discriminates between SSRIs differently than the hSERT, so this was used as an advantage to identify an allosteric site on the hSERT (Elfving & Wiborg, 2005; Larsen, Elfving, & Wiborg, 2004; Neubauer et al., 2006). Escitalopram and paroxetine bind 40-fold more potently to hSERT over gSERT, a difference not observed in other SSRIs, including fluoxetine and fluvoxamine, and not observed in serotonin's affinity for the two species of the SERT (Larsen et al., 2004).

This separation of SSRIs with potential allosteric activity from those without such activity led to mutagenesis studies to confer allosteric-like activity into the gSERT and out of the hSERT. Ultimately the escitalopram-hSERT binding profile was conferred into the gSERT and vice-versa, thus mapping an allosteric site (Neubauer et al., 2006). The allosteric site is in a distinct site from the previously mapped primary SSRI binding site (Zhong, Sánchez, & Caron, 2011). A mapped second locus for escitalopram binding at the hSERT supports the hypothesis that an allosteric site may be the cause of the measured clinical differences between citalopram and escitalopram.

After a second location for escitalopram binding was found, the next step was to identify the role of the site. Potentially the site was coincidental and did not explain the clinical separation between citalopram and escitalopram. That the hSERT binding profile could be conferred into the gSERT protein did support the hypothesis of an allosteric site, but it was not definitive support that this second binding location was able to affect the primary escitalopram binding in a modulating manner, as an allosteric site would affect an orthosteric site.

After identifying the allosteric site, *in vitro* work focused on the effects of the allosteric site on escitalopram's mechanism of action at the primary site. Changes in association and disassociation rates of escitalopram to and from the hSERT primary binding site were of particular interest. As previously noted, the hypothesis was that the allosteric site could alter the disassociation rate of escitalopram from the hSERT primary site, effectively causing escitalopram to inhibit the transporter for a longer period of time and causing an augmented buildup of extracellular serotonin (Figure 1). This serotonin could then continue its interactions outside of the cell for a longer period of time before

being recycled into the cell. Research found that escitalopram seemed to slow its own dissociation rate from the hSERT and that removal of the allosteric site decreased this effect (Chen, Larsen, Sánchez, et al., 2005; Chen, Larsen, Neubauer, et al., 2005; Neubauer et al., 2006; Zhong et al., 2009). Of course this work was *in vitro* and is not necessarily representative of how the system would act in a biological animal.

Purpose of Studies

The prior work studying escitalopram's allosteric binding site on the SERT is centered on identification of the locus. Because the allosteric site was identified by moving from clinical work to animal models, it is tempting to conclude that the allosteric site is the reason for the observed clinical phenomenon. However, that leap is not fully supported by the current body of literature at hand and remains a hypothesis. In order to properly make that link, the allosteric site's role itself must be studied. The site has no known endogenous ligand and there is no commercially available pharmacological compound without primary site activity. A new set of tools was necessary to further investigate the role of this putative allosteric site.

The work to map the allosteric site and confer allosteric activity into the gSERT resulted in identifying the key amino acids for escitalopram binding to the SERT. *In vitro* assays with mutant hSERTs identified that a series of three pairs of mutated amino acids eliminated the observed allosteric-like activity without significantly altering affinity of escitalopram for the primary binding site (Neubauer et al., 2006). Maintaining the same affinity for the primary binding site is an attractive quality in an allosteric-null hSERT construct because it allows for narrow study of the allosteric site without altering other actions of escitalopram at the hSERT. To more fully study the spectrum of activity the

allosteric site may facilitate, the allosteric-null hSERT was knocked into a mouse. The development of the knockin mouse will be discussed in depth in following chapters, but here it is of note the importance of the knockin mouse as an investigative tool. Mice are a well-studied model with an array of well-characterized assays to study the serotonergic system (Balu et al., 2013; Li, Morrow, & Witkin, 2006; Ripoll, David, Dailly, Hascoët, & Bourin, 2003; Strazielle, Lalonde, Riopel, Botez, & Reader, 1996). Escitalopram has been studied using mice which laid a baseline for allosteric investigation (Fish, Faccidomo, Gupta, & Miczek, 2004; Guiard, El Mansari, Murphy, & Blier, 2011; Sánchez & Kreilgård, 2004; Stórustovu et al., 2004; Thrivikraman et al., 2013). Additionally the genetic malleability of mice makes them an optimal tool for identifying the role of escitalopram's allosteric site at the SERT.

The central aim of the studies described herein was to identify the role of escitalopram's allosteric site on the SERT on the pharmacology of escitalopram using an intact animal model. These mice provided a novel tool for this work. For the first time *in vitro* work on the allosteric site and *in vivo* work studying escitalopram could be unified to accurately characterize escitalopram's allosteric activity. Previous *in vitro* work with the allosteric-null hSERT construct yielded answers about how escitalopram interacts with the hSERT. But this work lacks the ability to measure downstream effects that may affect an entire pathway, system or organism. Earlier work *in vivo* has characterized how escitalopram affects mice at pathway, system, and organism levels. The merging of the prior work onto the knockin mouse allowed for a focused examination of the escitalopram allosteric site. These mice were the first tools in which the cell line findings about the allosteric site were put to the test in an *in vivo* model. And in turn these mice

were the first tools to identify how the allosteric site activity may underlie known physiology and behavior in mice under an escitalopram challenge.

The allosteric-null hSERT mice were able to expand on prior work by illustrating if changes in the kinetics of escitalopram-hSERT have a functional output at the system level. The allosteric-null hSERT knockin mice also provided the necessary tool to pinpoint the allosteric site as the locus for escitalopram's different physiological and behavioral profile from that of citalopram. Initially these mice were for investigating escitalopram specifically, but they could reasonably be used in the future to investigate the influence of *R*-citalopram.

Ultimately the allosteric-null hSERT mice provided an invaluable link between the published work on the biochemistry of the allosteric site from *in vitro* work, to the physiology and behavior of escitalopram from *in vivo* work. In total, a full characterization of the allosteric site for escitalopram on the hSERT could be made. Not only an understanding of escitalopram's physical interactions at the site, but how that site plays a role in the neuropsychopharmacology of escitalopram in a living organism.

Aims and Hypotheses

The allosteric-null hSERT mice were ultimately a tool to study the *in vivo* role and value of the allosteric binding site for escitalopram on the hSERT. The first step was to evaluate the knockin mice as a tool for later experimentation. Determining how the knockin gene is expressed as well as its distribution in the mouse brain was the foundation to planning subsequent experiments to examine the functional role of the allosteric site. Autoradiography was the primary tool to do this, and was paired with veterinary observations of the knockin colony to define if the mice were a suitable tool.

After determining the value of the knockin mice as experimental tools, and identifying any allowances that should be made for deviations of the knockin mice compared to wildtype mice, then the physiological and behavioral experiments could be performed to evaluate the allosteric site. Simply hypothesized, more escitalopram would be required for allosteric-null hSERT knockin mice to achieve the same effect observed at a lower dose in mice with intact SERT (Figure 2). Additionally, this difference in potency due to the presence or absence of the allosteric site was not expected for SSRIs that have no allosteric activity. Overall the hypothesis was that the removal of the allosteric site on the hSERT will cause a decrease in escitalopram's potency in producing physiological and behavioral effects without altering escitalopram's efficacy. While there are hypothesized changes in escitalopram's potency, there are no expected alterations in potency or efficacy for SSRIs that do not bind the allosteric site, such as fluoxetine and fluvoxamine.

Figures

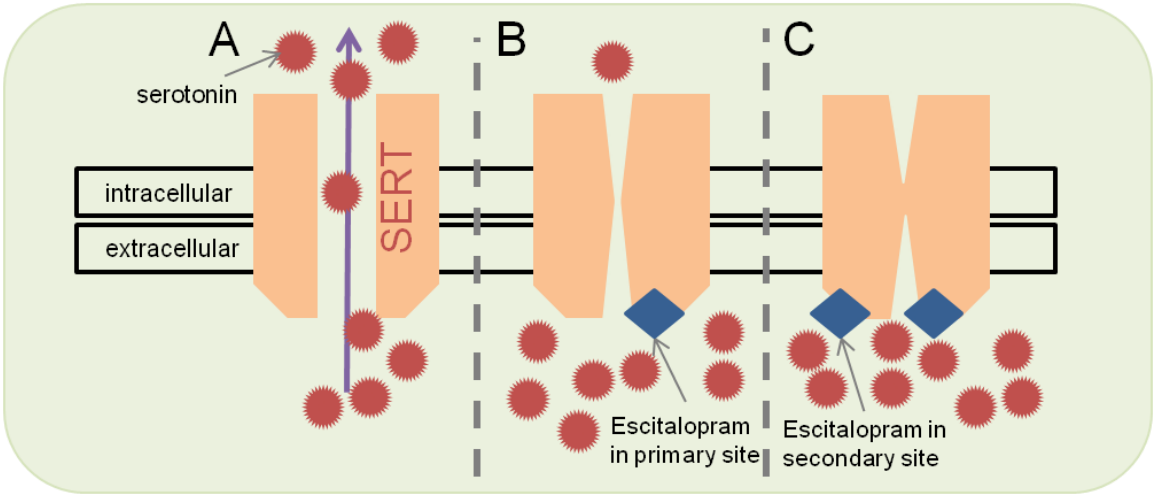

Figure 1: Hypothesis of escitalopram's activity at the SERT.

A) Without drug present the SERT moves serotonin into the cell. B) With escitalopram in the primary binding site the SERT is inhibited. C) With escitalopram in both the primary and the secondary (allosteric) binding sites the inhibition of the SERT is enhanced.

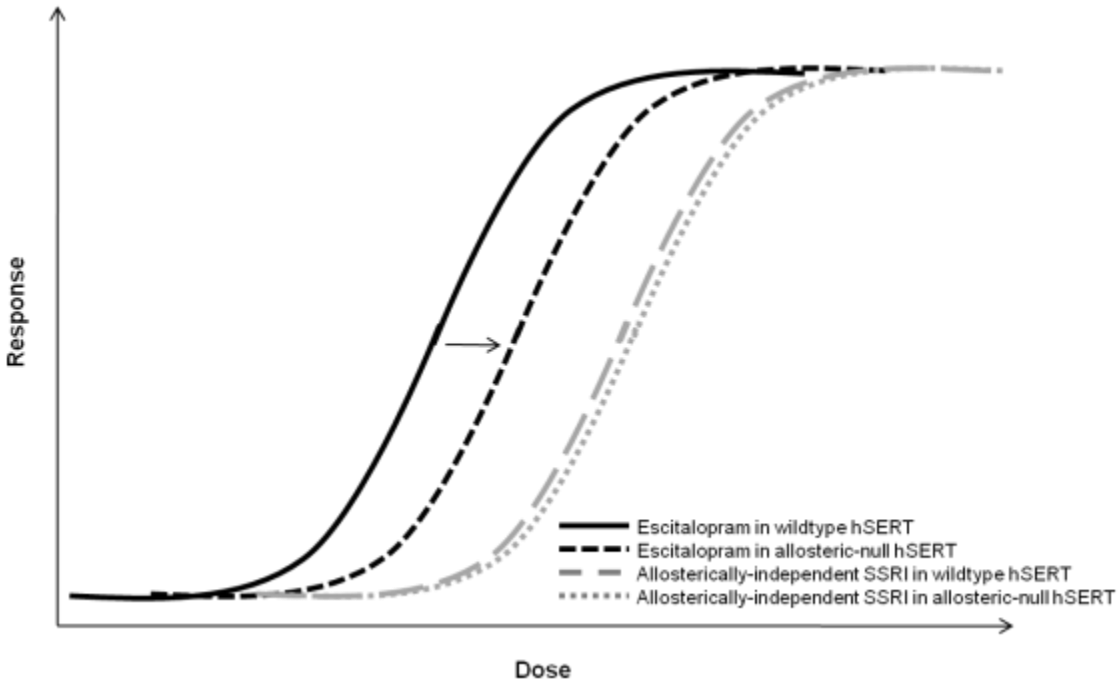

Figure 2: Theoretical dose-response curve of the hypothesized effect of escitalopram's allosteric site on the hSERT.

A rightward dose-response shift is hypothesized in the absence of the allosteric site, only for escitalopram but not for allosteric-independent SSRIs.

Chapter 2: Development of hSERT Mice

Necessity of hSERT Knockin Mice

In order to fully study the putative allosteric site for escitalopram on the SERT *in vivo* the proper tool had to be developed. A knockin mouse was thought and proposed to be the optimal tool. The malleability of the mouse genome provided for an anticipated straightforward development of a knockin animal. Other model organisms could have been receptive to the insertion of the desired hSERT gene, but organisms such as flies, worms and fish do not possess the complexity of a mammalian serotonergic system. And this makes the work with mice more translatable to the human serotonergic system, which as a mammalian system is more similar to the mouse system. The allosteric site was first mapped in the human transporter, and while the mouse transporter (mSERT) does display some allosteric activity (Fish et al., 2004; Sánchez & Kreilgård, 2004; Sánchez, 2006; Sánchez, Bergqvist, et al., 2003), it is ultimately the activity of escitalopram at the hSERT that is of particular interest. Escitalopram is a pharmaceutical agent meant for use in human patients, typically those suffering from a psychiatric illness. While it is possible that escitalopram could have veterinary use, the studies to be discussed herein are meant to fully understand the action of the compound at the human transporter. And because the development of knockin humans is neither a feasible or ethical tool for study, another model with a similar serotonergic system is desired. Mice fill the need more so than other species. Having a well-developed serotonergic system means that even though only the SERT is manipulated, it is not in isolation and thus the system is still working in concert with other systems. Therefore, measured alterations can be reasonably interpreted as caused by the manipulations. Seeing effects beyond the strict

molecular interactions renders results that are more translatable to the human system of ultimate interest. As previously discussed, a number of physiological and behavioral assays exist to study the serotonergic system in mice. Consequently, malleable genetics, existing serotonergic system, and an array of relevant assays make mice an appropriate tool to study the role of escitalopram's allosteric site on the hSERT.

Knockin Gene Construct

After identifying mice as the best model, the knockin gene construct was developed. The hSERT was inserted in place of the mSERT rather than manipulating the mSERT. The mSERT does display allosteric activity but it has not been fully characterized and mapped, as the hSERT has been. Previous *in vitro* work found that three pairs of point mutations resulting in three pairs of amino acid changes on the hSERT transmembrane domains 11 and 12 eliminated allosteric activity without altering primary escitalopram binding at the hSERT (Neubauer et al., 2006). These amino acid mutations are I522V/I553T/M558S/S559N/S574T/I575T. Importantly this location is distinct from the orthosteric binding site (Andersen et al., 2010). This set of mutations was used for the allosteric-null hSERT construct, referred to as hSERT-Emory. The hSERT-Emory construct was knocked into the mouse genome on chromosome 11, removing the mSERT gene (See Appendix A for more about the knockin construct and insertion). The resulting knockin eliminates the mouse transporter so that the mouse only has the human gene. This means that only the hSERT construct is expressed and that any SERT activity measured can only be attributed to the hSERT, and not to the mSERT. The hSERT-Emory construct is expressed under the mSERT promoter region in order to preserve the mouse machinery to transcribe, translate and express the hSERT protein in

place of the mSERT. As a control for the genetic manipulation, a parallel knockin mouse with native, or wildtype, hSERT (hSERT-wt) was created. This construct serves as a control to the hSERT-Emory to directly compare the effect of eliminating the allosteric site in comparison to a similar humanized knockin construct. Comparing knockin hSERT-Emory mice to wildtype mice with mSERT is not an appropriate comparison due to humans and mice not having 100% homology at the SERT. Furthermore, in all knockin manipulations there is the possibility that removing the intronic sequence will alter the genetic machinery's ability to create and express the protein, especially a gene from another organism. The hSERT-wt knockin mice provide a necessary safeguard that enhances the tool of the hSERT-Emory knockin mice to accurately and effectively study the allosteric site in an all-humanized SERT genetic background.

Production of the hSERT colony

The production of the hSERT-wt and hSERT-Emory mice was completed in collaboration with Marc Caron's group at Duke University (Durham, NC). The sequence of the hSERT-wt and hSERT-Emory construct was confirmed by Kerry Ressler (Emory University). See Appendix A for full sequence and insertion protocol. The two hSERT constructs were created via homologous recombination into the mSERT locus with 129S6 embryonic cell lines and implanted into 129S6/SvEvTac (Taconic) mice. Following confirmation of site specific recombination, each line was backcrossed to establish to homozygosity and shipped from Duke University to Emory University. Pilot work found that C57BL/6J (Jackson Labs) mice were more suitable for the planned behavioral experiments. Consequently the homozygous hSERT-wt and hSERT-Emory lines were backcrossed 10 generations from 129S6/SvEvTac into C57BL/6J. After the

backcross, the mice were once again bred to homozygosity and the establishment of the two knockin lines was complete. The two lines were then crossed to produce heterozygous hSERT-wt/Emory mice. These mice are the ideal breeders for studies of the escitalopram allosteric site because a heterozygous breeding pair will produce littermate 25% hSERT-wt, 25% hSERT-Emory, and 50% hSERT-wt/Emory pups. Overall this increases the similarity between the hSERT-wt and hSERT-Emory mice and decreases possible parental effects or *in utero* effects on biology and behavior. And this maximizes similarity between hSERT-wt and hSERT-Emory mice so that differences between these lines may be attributed to the allosteric site genetic differences.

The colony of hSERT mice displayed no gross differences from wildtype C57BL/6J mice. The mice are able to breed normally, producing typical-sized and healthy litters. Emory University's Division of Animal Resources (DAR) has noted no unusual health differences between these knockin mice and wildtype mice, making the mice suitable for the rigors of experimentation. Individual mice are genotyped using allele-specific primers that can differentiate between the two hSERT constructs (full details in Appendix B). The hSERT colony has been maintained for multiple generations, suitably producing the necessary number of mice for pilot and experimental study.

Maintenance of the hSERT Colony

All hSERT knockin mice were housed in vivariums managed by Emory University's DAR. Breeder animals were kept in pairs or trios, and were completely managed by the DAR Managed Breeder Colony. Tail clips were provided for genotyping, but pairings and weaning were overseen by DAR staff. Upon weaning animals were moved out of the DAR Managed Breeder Colony, and were group housed by sex, up to

five mice per cage. At all points the mice had *ad libitum* access to mouse chow pellets and water, with a 12:12 light:dark cycle, with the light cycle beginning at 7:00 am.

For some pilot studies wildtype C57BL/6J mice were used (Jackson Laboratories). These mice were used to test for feasibility of assays, rather than as comparators to the hSERT knockin mice. Once at Emory, the mice were treated and cared for in the same manner as the hSERT knockin mice, with same access to food and water and same light cycle.

Chapter 3: Autoradiography

Abstract

When developing a knockin mouse model it is important to characterize the expression of the knockin gene. An ideal construct would express only where desired and at the desired level. In this case, the hSERT-wt or hSERT-Emory knockin protein would replicate the location and levels of mSERT expression in the mouse brain. The expression of the SERT was mapped using [^{3}H]-citalopram autoradiography in knockin mice, hSERT-wt, hSERT-Emory and hSERT-wt/Emory, and mSERT mice. The frontal cortex, hippocampus, and raphe nucleus were examined. Overall, SERT expression was decreased in the knockin lines compared to wildtype mice, reaching a statistically significant level in the frontal cortex and the raphe nucleus. However, in all three regions there was no statistical difference in hSERT expression between the three knockin lines. These findings characterize the knockin mice as useful tools, with possible limitations, to model the role of escitalopram's allosteric site on the SERT.

Introduction

Expression of the knockin hSERT gene as a protein is important to map and quantify. Although the hSERT genes are under the endogenous mSERT promoter region, the ultimate translation of the DNA into protein could be affected by the differences between the mouse and human genes. Additionally, the differences between the species' proteins could cause changes in translocation or expression in cells. Even if there are no effects of inserting a knockin gene, the removal of the allosteric site could alter how the hSERT-Emory gene is translated and expressed compared to the hSERT-wt. Although there is no identified endogenous ligand for the escitalopram allosteric site on the SERT, it is possible that the removal of the site inhibits the cellular machinery to properly express the protein in the cell surface. Altered hSERT expression in the mice could affect the ability to directly compare the hSERTs to each other or to use the knockin mice as a tool to understand the interaction of escitalopram at the SERT.

Autoradiography is an excellent tool to map SERT expression in the brains of the knockin mice. The technique allows for visual mapping of the location of the SERT protein as well as quantitative means to compare the knockin lines to each other as well as to wildtype mice. Using [^{3}H]-citalopram as a highly selective radioligand marker of the hSERT protein is a good proxy for the level of hSERT insertion in neurons.

The frontal cortex, the hippocampus, and the raphe nucleus were chosen as representative regions for the autoradiographical mapping. Each of these regions is known to have SERT expression in wildtype mice (Jennings et al., 2006; Le Marec, Hébert, Amdiss, Botez, & Reader, 1998; Strazielle et al., 1996). The frontal cortex and hippocampus each receive serotonergic inputs while the raphe nucleus is the central

nucleus of serotonergic cells. Serotonergic pathways are found throughout the mouse brain, but these three regions are well-defined regions with well-mapped serotonergic roles. The hippocampus is also the location of microdialysis experiments to examine the physiological effects of removing the allosteric site from the SERT (Chapter 4). Any differences between the knockin lines or compared to wildtype mice could be telling in the analysis of the microdialysis work. Overall, a difference in expression between the knockin lines could be telling in the interpretation of any physiological or behavioral results using these mice. A difference in the hSERT expression could be the underlying reason for physiological and behavioral effects, rather than being able to pinpoint an effect to the allosteric site.

In sum, the autoradiography to map SERT expression in hSERT-wt, hSERT-Emory and mSERT mice is an essential step in both characterizing the knockin mice as well as laying a foundation to understanding how escitalopram interacts with its allosteric site on the SERT. Some differences between mouse lines could be compensated for in a physiological or behavioral assay by adjusting drug challenge dosing. A variation in knockin gene expression compared to wildtype mice might alter the expected physiological and behavioral results, but knowing how the hSERT-wt and hSERT-Emory proteins compare is essential to verifying these mice as viable tools for their intended work.

Materials and Methods

To make the necessary comparisons between the hSERT-wt, hSERT-Emory, and mSERT expression in mouse brains, tissue was collected from mice of each of those genotypes, as well as from heterozygous hSERT-wt/Emory mice. The heterozygous mice

were included because these mice were to be used in pilot experiments for physiology and behavior experiments, and knowledge of their SERT expression would we valuable in identifying them as appropriate pilot mice. Similarly, both male and female mouse brains were included so that both sexes could be characterized. Mice were euthanized with an overdose of isoflurane and then quickly decapitated. Immediately the brain was extracted and fresh frozen on dry ice. All brains were stored at -80 °C until use.

Brains were sliced at -16 °C on a cryostat at 20 µm thick slices. Serial sections were distributed across four series, with 4 slices obtained at the level of the frontal cortex, hippocampus and raphe nucleus. Multiple series of the slides allowed for total binding and non-specific binding to be performed in the same brains at near identical slices. Non-specific binding was a control to account for any background binding, rather than comparing to a control region with low SERT expression. Regions were identified according to Franklin and Paxinos's The Mouse Brain in Stereotaxis Coordinates (Franklin & Paxinos, 1997). Frontal cortex slides were at Figure 22, hippocampus slides at Figure 47, and raphe nucleus slides at Figure 68, following the atlas. Slices were fixed on Fisherbrand Superfrost/Plus Microscope Slides (Catalog No. 12-550-15), and were stored at -80 °C until used in the autoradiography.

The radioligand for the autoradiography was [^{3}H]-citalopram. The racemic compound was chosen over enantiopure escitalopram due to commercial availability of radioligand. An escitalopram radioligand would have also been appropriate, but no difference in binding affinity between the hSERT-wt and hSERT-Emory constructs *in vitro* indicates that any differences caused by the removal of the allosteric site would not affect the primary site binding. Consequently, for the purposes of the autoradiography

studies, citalopram, escitalopram or another SERT-selective ligand are effective radioligands.

Series 1 and 2 of the brain slices were used for autoradiographic experiments, with series 1 for total binding and series 2 for non-specific binding. Brain slices were thawed to room temperature then preincubated in SERT buffer (52.2 mM Tris HCl, 126.4 mM NaCl, 5.26 nM KCl) for 15 minutes. Total binding and non-specific binding solutions were also made in SERT buffer. The total binding solution was 2 nM [^{3}H]-citalopram, and the non-specific binding solution was 2 nM [^{3}H]-citalopram with 1 µM escitalopram. The high concentration of escitalopram would be able to out-compete the tracer concentration of citalopram radioligand, and any measured bound radioligand could be subtracted as non-specific binding. The concentration of 2 nM [^{3}H]-citalopram was chosen because it is similar to the K_d of citalopram for the hSERT and the mSERT (Owens et al., 2001). The slides incubated in either the total binding or non-specific binding solutions for 60 minutes. The slides were then washed 2 by 10 minutes in 0 °C SERT buffer to end the exposure to the radioligand and in the case of the non-specific binding the competitive escitalopram. Slides were then dipped in cool dH_2O and finally dried under a cool stream of air. The slides air dried for several hours to make sure they were fully dry prior to exposure to film.

Pilot work using mSERT brain slices to determine the appropriate exposure time indicated that the brain slices needed to be exposed to the film for 12-13 weeks. Once the slides were fully dry, they were placed in a cassette and exposed to Kodak Biomax MR Film for 13 weeks. A tritium standard was included in the cassette for quantitative measurements. After 13 weeks the film was developed.

The NIH's ImageJ was used to make quantitative densitometric measurements. For each mouse one slice at each the frontal cortex, hippocampus, and raphe nucleus was analyzed. In the ImageJ program the tritium standard was used to calibrate a standard curve. For each region a free-hand shape was drawn to outline the region, and ImageJ determined a density which was then converted to nCi/mg based on the standard curve. An identical shape was used to determine the nCi/mg for matching total binding and non-specific binding slides. Non-specific binding was subtracted from total binding, and then analysis was performed using IBM's SPSS. A 1-way ANOVA was used to compare the nCi/mg values of SERT expression across the four genotypes of hSERT-wt, hSERT-Emory, hSERT-wt/Emory, and mSERT.

Results

Upon visual inspection of the film it was obvious that the mSERT mice expressed the SERT protein more than any of the knockin lines. This qualitative analysis was supported by the quantitative analysis in the frontal cortex and the raphe nucleus, although there was no significant difference in the hippocampus (data discussed below, values in Table 1). Overall, this indicated that the knockin protein expresses less readily than the endogenous mSERT. Importantly, there were no significant differences in SERT expression between the hSERT-wt and hSERT-Emory mice at any of the three measured regions.

In the frontal cortex there was a significant effect of genotype on SERT expression, as measured in nCi/mg, $F(3,24) = 4.251$, $p = 0.017$ (Figure 3). Post hoc test determined that in mSERT mice the SERT expressed significantly more than in hSERT-wt and hSERT-wt/Emory mice ($p < 0.05$ for both), and trended towards increased

expression compared to hSERT-Emory mice ($p = 0.052$). There was no significant difference between the knockin lines.

In the hippocampus there was no significant difference in SERT expression across then genotypes, $F(3,24) = 0.927$, $p = 0.445$ (Figure 4). The absolute value of nCi/mg measured in the mSERT is greater than any of the knockin lines, but this did not reach significance. Additionally, it is important to note that there is no difference between the knockin lines, as well.

In the raphe nucleus there was a significant effect of genotype on expression of the SERT, $F(3,24) = 4.615$, $p = 0.012$ (Figure 5). Post hoc tests identified mSERT mice as expressing significantly more SERT compared to hSERT-wt and hSERT-wt/Emory mice ($p < 0.05$ for both), and trended towards an increase over hSERT-Emory mice ($p = 0.051$). There were no significant differences in expression between the knockin lines.

Conclusions

The autoradiography assay was to identify if the knockin mice were expressing the hSERT proteins, if expression varied compared to mSERT expression, and if there were any differences in expression between the hSERT-wt and hSERT-Emory mice. Secondary was answering if the hSERT-wt/Emory mice were similar to the homozygous mice and thus appropriate for pilot work to study the hSERT-wt and hSERT-Emory mice. Answering these questions would characterize the hSERT knockin mice and define how they could best be used in physiological and behavioral studies to examine escitalopram's allosteric site on the SERT.

First, the autoradiographic studies confirmed expression of the knockin hSERT in the brains of mice. Specifically, bound radioligand was measured in each the frontal

cortex, hippocampus, and raphe nucleus, indicative of SERT protein in those regions. This was essential to characterizing the mice as tools for studying escitalopram-hSERT interactions. Had there been no SERT expression in regions where SERT is known to be in the mouse brain this would have indicated either a failure in inserting the gene or that the human protein could not be properly expressed in a mouse.

Second, there is a decrease in expression of the knockin proteins compared to endogenous mSERT. This decrease reaches statistical significance in the frontal cortex and raphe nucleus, but not the hippocampus. Overall, decreased hSERT expression compared to mSERT suggests that in assays that rely on SERT activity there are likely to be differences between hSERT and mSERT mice. The hSERT-wt and hSERT-Emory mice are meant for specifically understanding the role of escitalopram's allosteric site on the hSERT, and no direct comparisons to mSERT mice are intended. Yet, this would suggest that any assay used may have modulated outcomes compared to other work completed in wildtype mice, and should be considered when designing studies with these knockin mice.

Finally, there is no difference between the hSERT-wt and hSERT-Emory expression in any of the three regions. Also, hSERT-wt/Emory mice do not have altered expression compared to the other knockin lines. This is a key finding in characterizing the knockin lines for use to study the allosteric site. A difference in expression between the hSERT-wt and hSERT-Emory mice would decrease their value in studying the allosteric site. Any measured physiological or behavioral differences between the lines could not be pinpointed to the presence or absence of escitalopram's allosteric site. Rather it would be ambiguous if differences between the hSERT proteins activity was

due to the allosteric site or the level of hSERT expression, and there would not be a simple method to differentiate the two. Similar hSERT expression between the knockin lines confirms they are the right tool to use. Additionally, that there is no difference in expression between the homozygous lines and the heterozygous line further indicates that the hSERT-wt/Emory mice are useful tools for pilot experiments. Their decreased SERT expression compared to mSERT mice would account for the same decrease in the homozygous mice, without providing a bias towards one allele when developing a method.

Figures

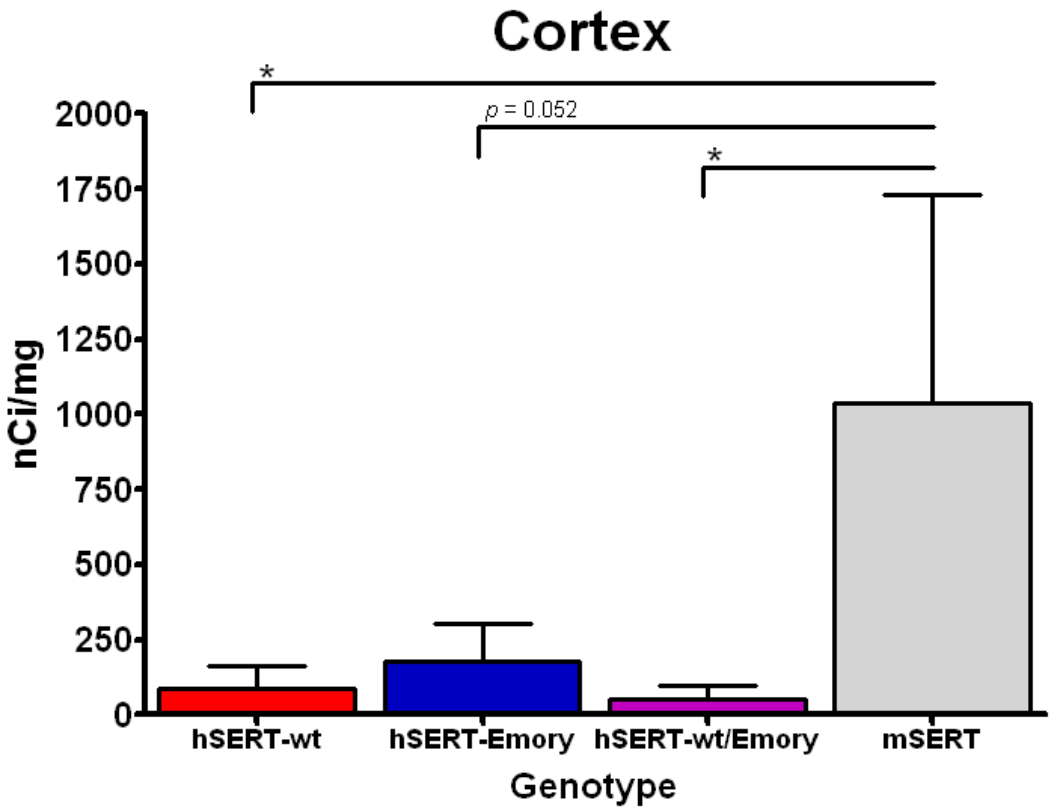

Figure 3: Measured expression of SERT in the cortex.

Significant effect of genotype, $F(3,24) = 4.251$, $p = 0.017$, with hSERT-wt mice and hSERT-wt/Emory mice expressing significantly less SERT than mSERT mice ($p < 0.05$) and a trend of decreased expression in hSERT-Emory mice ($p = 0.052$). Mean ± SEM.

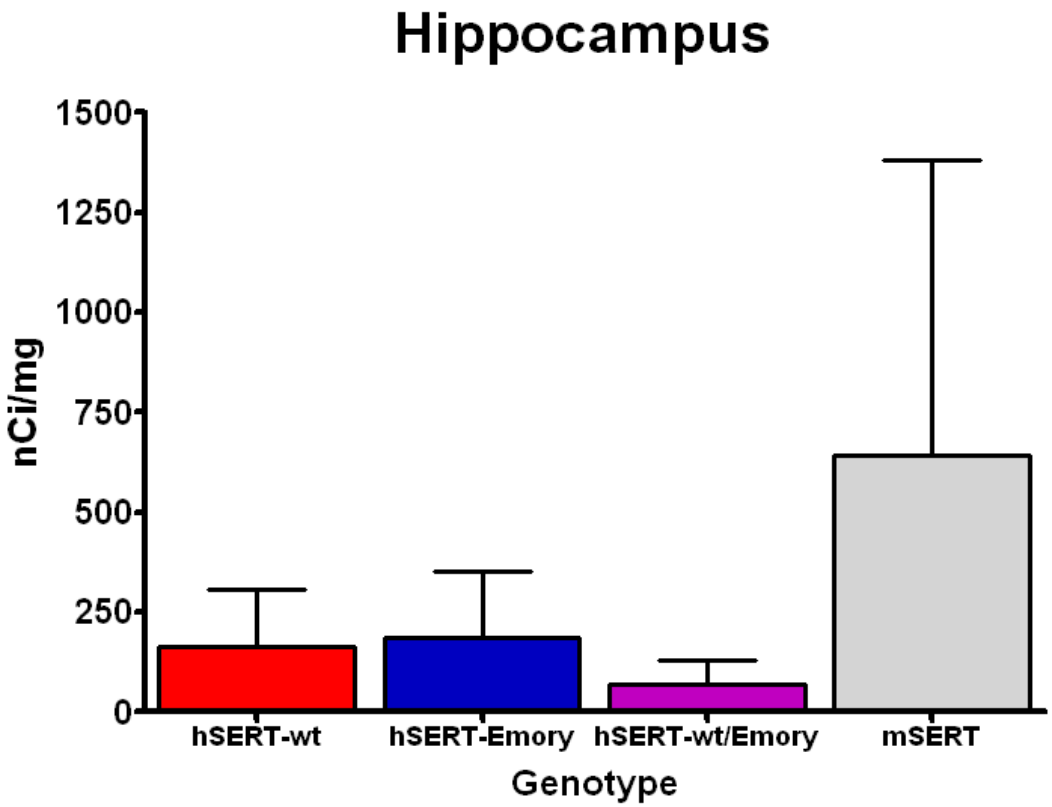

Figure 4: Measured expression of SERT in the hippocampus.

No significant effect of genotype, $F(3,24) = 0.927$, $p = 0.445$. Mean ± SEM.

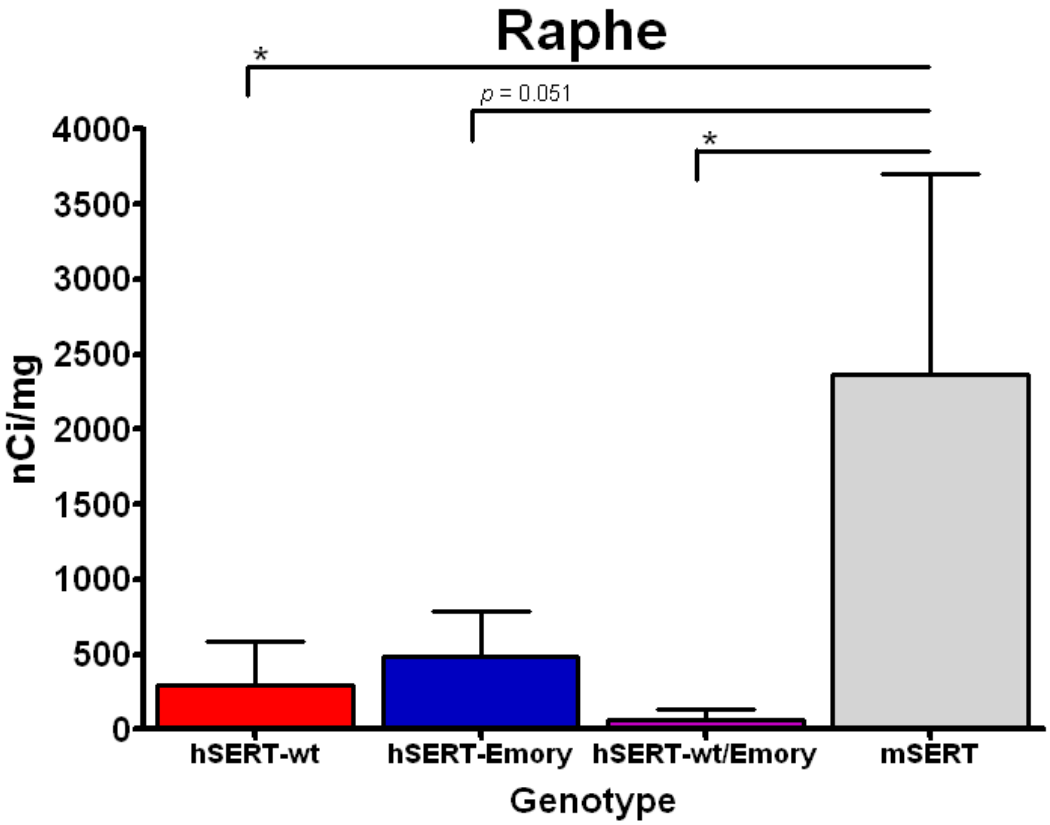

Figure 5: Measured expression of SERT in the raphe nucleus.

Significant effect of genotype, $F(3,24) = 4.615$, $p = 0.012$, with hSERT-wt mice and hSERT-wt/Emory mice expressing significantly less SERT than mSERT mice ($p < 0.05$) and a trend of decreased expression in hSERT-Emory mice ($p = 0.051$). Mean ± SEM.

Table

nCi/mg (mean ± SEM)	hSERT-wt (n=7)	hSERT-Emory (n=7)	hSERT-wt/Emory (n=8)	mSERT (n=3)
Cortex	87.20 ± 73.83	176.7 ± 126.2	53.76 ± 45.68	1038 ± 692.4
Hippocampus	163.9 ± 141.2	186.9 ± 166.3	68.92 ± 61.09	640.4 ± 741.8
Raphe	297.4 ± 289.3	481.5 ± 309.4	65.61 ± 64.77	2361 ± 1336

Table 1: Autoradiographic measurements in the mouse brain.

SERT expression is reduced in the knockin lines in the cortex and raphe nucleus, $p < 0.05$.

Chapter 4: Microdialysis

Abstract

Microdialysis studies were performed to measure the physiological role of the allosteric site in escitalopram action. Microdialysis in the mouse ventral hippocampus measured baseline serotonin levels as well as response to retrodialysis application of escitalopram into the region. Baseline serotonin levels were the same for both genotypes. Escitalopram evoked a significant increase in extracellular serotonin at both 0.0462 µM and 0.1 µM, but there was no difference in extracellular serotonin response between the low and high concentration drug challenge. Both the peak response as well as the cumulative response over time was analyzed to identify if there was a difference due to the presence or absence of the allosteric site. There was no significant genotype effect on peak response nor on response over time. The microdialysis data do not support an *in vivo* role of the allosteric site on escitalopram activity in the ventral hippocampus.

Introduction

To understand the *in vivo* effects of escitalopram's allosteric site on the hSERT it is important to measure how the physiological effect of escitalopram is altered by the presence or absence of the allosteric site. Based on *in vitro* data, the hypothesis is that allosterically bound escitalopram causes primary site bound escitalopram to inhibit the hSERT longer, resulting in a buildup of extracellular serotonin (Larsen et al., 2004; Neubauer et al., 2006; Plenge & Mellerup, 1997; Stórustovu et al., 2004). Removing the allosteric site could consequently blunt the amount of extracellular serotonin buildup because the transporter will be able to transport serotonin sooner as inhibition is shorter. This difference in extracellular serotonin levels could be measured by microdialysis in the brain. Microdialysis allows for local measurement of molecules in living brains and is a measurement of the physiological effects of the presence or absence of the allosteric site.

Previous work indicates that the extracellular increase in serotonin due to escitalopram can be measured in the brains of rodents (Bundgaard, Jørgensen, & Mørk, 2007; El Mansari et al., 2007; Mørk et al., 2003; Thrivikraman et al., 2013). Much of this work was in rats, but it laid groundwork that the physiological role of escitalopram can be measured *in vivo*. Specifically, the work of Mørk et al. (2003) was pivotal in identifying the existence of an allosteric site. Looking the SERT-rich frontal cortex, the group found that subcutaneous injections of escitalopram resulted in an increase of extracellular serotonin, an effect that was blunted by a co-administered injection of *R*-citalopram. The blunting effect of *R*-citalopram was also replicated upon retrodialysis infusion of the two citalopram enantiomers into the rat frontal cortex. Of importance is the percentage

increase over baseline after systemic escitalopram challenge and via retrodialysis was approximately 400-500%, suggesting that the 0.5 µM escitalopram concentration infused by retrodialysis was similar to what the frontal cortex received after 2.0 mg/kg subcutaneous injection.

This prior rat work focused on examining the interaction of citalopram's two enantiomers and built support for the hypothesized allosteric site. Since the identification of the allosteric site it became clear that it was also necessary to examine escitalopram alone at the site, not just the interaction of it with its racemate. While the interaction is interesting, it does not elucidate what is happening with escitalopram alone that could then be translated in other compounds that could target the site specifically to enhance primary acting compounds on the hSERT. The hSERT-wt and hSERT-Emory knockin mouse lines allow for the necessary physiological investigation of escitalopram's allosteric site on the hSERT.

In preparation for the knockin mouse studies, Thrivikraman et al. (2013), developed a method for microdialysis in the mouse with in-line retrodialysis. The method is described below in detail, but it was tested using escitalopram in C57BL/6J wildtype mice. As much of the prior work examining escitalopram was completed in rats, it was necessary to confirm that serotonin could be measured in the mouse brain and that escitalopram evoked a measurable response. In an anesthetized mouse, microdialysis in the ventral hippocampus reliably sampled serotonin. Additionally, Thrivikraman et al. found that both subcutaneous and retrodialysis administration of escitalopram evoked an increase in extracellular serotonin levels in the ventral hippocampus of the mice. In the retrodialysis experiments the serotonin levels did start to return to baseline after initial

challenge but remained elevated 1 hour after escitalopram exposure. A second infusion of the same concentration of escitalopram 1 hour later also evoked an additional response. Although the second drug challenge occurred before serotonin levels returned to baseline and no true quantitative measures could be made, qualitatively the ability to evoke a second response suggested that a dose-response curve may be possible within a single mouse.

The ventral hippocampus is a reasonable location for the microdialysis experiments. The hippocampus receives large amounts of serotonergic input, resulting in expression of the SERT and measurable serotonin levels. This is also true of the frontal cortex, as was studied in rats by Mørk et al. (2003), but practically the dimensions of the ventral hippocampus in the small mouse brain makes it a more feasible target for study. There could be region differences in action of local infusion of escitalopram, but the ventral hippocampus is a proven and practical place to begin *in vivo* microdialysis studies. Using the method developed by Thrivikraman et al., microdialysis, with retrodialysis of escitalopram, in the ventral hippocampus examined the physiological role of escitalopram's allosteric site with side by side comparison of the hSERT-wt and hSERT-Emory mice.

Materials and Methods

Microdialysis was modeled after a published method which allows for microdialysis sampling as well as retrodialysis application of escitalopram in the same location (Thrivikraman et al., 2013). This method is verified for use in mice, for the measurement of serotonin, and the application of escitalopram, making it an appropriate method to compare the hSERT-wt and hSERT-Emory mice. Pilot studies were completed

to determine escitalopram concentrations to use as well as to confirm the use of fluoxetine as an allosteric-inactive SSRI control. The published method identified that a second retrodialysis application of escitalopram could evoke a second response, however it was clear that 1 hour after initial drug exposure was not enough time to return serotonin levels to baseline concentrations. Pilot work identified 2 hours as an appropriate time-frame to separate drug challenges in both escitalopram and fluoxetine. After 2 hours the measured serotonin levels returned to baseline levels. Pilot work also indicated that repeating the same drug concentration in 2 hour intervals resulted in similar responses between the two drug challenges. Additionally, if the second drug challenge was of a higher concentration, the pilot work indicated a larger response to the larger drug concentration. All pilot work was completed in hSERT-wt/Emory mice to account for any serotonergic system changes that may occur with the decreased hSERT expression, as found in the autoradiography studies (Chapter 3). The final concentration of escitalopram and fluoxetine was the same: 0.0462 μM for the low concentration and 0.1 μM for the high concentration, a one-third log-step separation. These concentrations were determined empirically through pilot testing, which showed that both concentrations evoked a response over baseline. Pilot testing also indicated that the higher concentration caused a larger response than the lower concentration.

Together the pilot work defined the modifications to the Thrivikraman et al. method so that the *in vivo* physiological role of escitalopram's allosteric site could be measured. All microdialysis studies were completed in anesthetized mice, and the entire procedure lasted 8 hours, with the mouse being decapitated before recovering from anesthesia so that its brain could be collected. All procedures began between 8:00 am and

10:00 am. Mice were anesthetized with urethane (Sigma-Aldrich), at 1.6 g/kg delivered in at volume of 15 µL/g. Each mouse was weighed so that a proper dose of urethane was delivered. Once the mouse failed to react to a firm toe-pinch, it was secured in the stereotactic frame. The top of the mouse's head was cleaned with 70% ethanol and an incision was made with a clean scalpel to reveal the skull, which was also cleaned with 70% ethanol. Relative to bregma, using a small-bore drill, a hole was made at in the skull at AP = -3.2 and ML = -2.5. The membranes were slit with a needle and the site was dabbed with a cotton swab until any bleeding subsided. Using an adaptor to the stereotax and a probe clip, the microdialysis probe was aligned with the center of the hole in the skull. The tip of the probe was then inserted to DV = -4.0. The coordinates of AP = -3.2, ML = -2.5, and DV = -4.0 placed the probe within the ventral hippocampus, approximately at Figure 57 in The Mouse Brain in Stereotaxis Coordinates (Franklin & Paxinos, 1997). The probe was then secured in place using dental cement. After the cement was firm and dry the mouse was carefully placed on a heating pad for the remaining duration of the microdialysis.

The microdialysis probe was a CMA 11 (Harvard Apparatus). The cuprophane probe had a 6 kDa cutoff. The probe length was 2 mm. Some pilot work was completed with 3 mm probes, matching the published work. The switch to 2 mm was made so that the entire exposed probe length would more reliably and consistently be completely within the ventral hippocampus. With the entire mouse brain being 5-6 mm thick at this level, there was concern that the 3 mm probe may be measuring more than the desired ventral hippocampus. A 2 mm probe was determined to sample serotonin just as reliably, without concerns of measuring beyond the intended region.

Prior to insertion, the microdialysis probe was primed overnight. This priming removed the storage glycol from the probe and equilibrated it to the artificial cerebrospinal fluid (aCSF, 142 mM NaCl, 2.95 mM KCl, 1.36 mM $CaCl_2 \cdot 2H_2O$, 0.98 mM $MgCl_2 \cdot 6H_2O$, 0.99 mM Na_2HPO4, 0.22 mM $NaH_2PO_4 \cdot H_2O$). To prime the probe, it was attached to the tubing of the microdialysis system and inserted in a 1.5 mL tube filled with aCSF. The microdialysis pump was turned on so that a flow rate of 1 µL/min was maintained. This flow rate was maintained throughout the duration of the priming and through the duration of the experiment with the mouse. Once the probe began priming the flow rate never ceased until the experiment was completed, and the probe was attached to the microdialysis system the entire time. Prior to insertion into the mouse and after priming, serotonin recovery was measured for each probe, with the probe being placed in a tube of 5 nM serotonin (as serotonin HCl, Sigma-Aldrich). A sample was collected and compared to 5 nM serotonin fresh from the tube, along with the rest of the samples.

The microdialysis system was unaltered from the published Thrivikraman et al. study, and an overview of the system can be seen in Figure 6. In brief, a 6-port valve (Upchurch) was used to regulate the switch between a flow of aCSF or aCSF with SSRI for retrodialysis. A schematic of the valve can be seen in (Figure 7). The valve allows for two simultaneous pathways. When in the LOAD configuration, aCSF flows directly through the valve, bypassing the loop, heading through the probe and out to the sample collection. In the other pathway the loop could be filled, in the case of these studies the loop was filled with either escitalopram oxalate or fluoxetine HCl solutions. When in the INJECT configuration, the loop is now part of the pathway to the probe, thus allowing for any compounds in the loop to be applied through the probe via retrodialysis. The tubing

for the loop, the connection of the valve to the probe, and from the probe to sample collection each had a volume of 20 µL. With a flow rate of 1 µL/min, every 20 minutes a sample was collected of a volume of 20 µL. This allowed a collected sample to match the exact exposure time of the solution filled into the loop. Here this allowed for a single sample to match the exposure to either escitalopram or fluoxetine. Samples were collected into HPLC vials and were immediately capped and stored at -80 °C until analysis via HPLC (described below). Flow was regulated with an infusion pump (Fisher), using 5 mL plastic syringes as the stock of aCSF that flowed from the beginning of priming through the end of the experiment, approximately 24 hours.

A schematic of the experimental timeline is found in Figure 8. After the microdialysis probe is inserted into the mouse's ventral hippocampus, there is an equilibration and recovery period of 80 minutes (4 samples). This time allows mouse's brain to recover from the insult of inserting the probe and no samples were analyzed from this time. Analyzed pilot samples indicated that serotonin concentrations were extremely high during this time, but leveled out after this recovery time. After the recovery, time was set to 0 minutes and the first baseline sample was labeled 0 minutes (consequently sample collection lasted from -20 to 0 minutes). Four baseline samples were collected, every 20 minutes, a pattern maintained for the duration of the experiment. The first drug challenge sample was from 60-80 minutes, and labeled sample 80 minutes. The second drug challenge was from 180-200 minutes, and labeled sample 200 minutes. A third challenge from 300-320 minutes was of a high potassium concentration (called high K+, aCSF with 10X KCl). This high K+ challenge was to determine the integrity of the tissue because it should evoke a release of neurotransmitters, including serotonin, unless the

tissue was compromised. Low high K+ response was criteria for removal a mouse from the data set. At 360 minutes the probe was removed, rinsed in aCSF, and then placed in 5 nM serotonin for post-experiment serotonin recovery, as was done prior to inserting the probe. Each mouse received a low concentration followed by a high concentration of the either escitalopram or fluoxetine, and no mouse received both SSRIs.

After the probe was removed, the mouse was decapitated, its brain removed, and fresh frozen on dry ice. The brain was stored at -80 °C for later analysis of proper probe placement. Any misplaced probe resulted in removal of that mouse from the data set. Any mouse that died during the microdialysis experiment was also excluded. If a mouse needed a booster of the urethane it could be completed only during the probe insertion recovery or baseline sample collection. The individual sample that matched the time of booster injection was removed, leaving 3 baseline samples instead of 4. This was done because the injection resulted in a rapid spike in serotonin levels only in the matched sample.

High-performance liquid chromatography (HPLC) was used to measure the serotonin in the microdialysis samples. Each mouse had 4 baseline samples, 6 samples from low concentration of SSRI, 6 samples from high concentration of SSRI, 3 samples from high K+, 4 samples for probe serotonin recovery (2 pre-experiment, 2 post-experiment), a total of 23 sample vials per mouse. All samples per individual mouse were run in a single batch run on the HPLC with appropriate standards. The serotonin standards were 0.1, 0.2, 1, 2, and 5 nM, and created a standard curve to quantify the serotonin in each sample.

The HPLC system was composed of C18 column (ESA), dimensions of 150 X 3.2 mm with a 3 µm particle size and 120 Å pore size. MD-TM mobile phase (90 mM NaH_2PO_4, 50 mM monohydrate citric acid, 1.7 mM 1-octanesulfonic sodium salt, 50 µM EDTA, 10% acetonitrile, pH = 3, made in ddH_20) was delivered through the system via an ESA 528 solvent delivery pump, set at a flow rate of 0.600 mL/min. Samples were loaded into the ESA Model 542 refrigerated autosampler, 7 °C. The autosampler loaded 15 µL of the individual 20 µL dialysate sample onto the column. An ESA Coulochem III detector with a model 5040 ESA dual-channel analytical cell and model 5020 ESA guard cell were used for electrochemical analysis. EZChrom Elite software (Scientific Software) on a desktop computer collected the data and created the chromatograms. The EZChrom Elite software was also used to analyze and quantify the data using the created standard curve for each batch run. The standard curve resulted in determination of the serotonin concentration for each individual sample.

Data for each mouse were analyzed in two ways: percent change from baseline and area under the curve (AUC) for a plot of serotonin concentration over time. Percent change from baseline analysis illustrated the peak response after each drug challenge, allowing for conclusions about if the absence of the allosteric site in hSERT-Emory mice resulted in a blunted magnitude of effect compared to hSERT-wt mice. AUC analysis allows for examination of a change in drug response over time due to the presence or absence of the allosteric site. Together these data can give a more robust picture of any genotype effect.

In the percent change from baseline analysis, for an individual mouse, the serotonin concentration in the 4 baseline samples was averaged, and then the serotonin

concentration for all samples were converted to a percentage of the average baseline value. The peak response for each the low and high drug concentrations challenge was then analyzed. SPSS (IBM) was used to run a 2-way ANOVA to determine the genotype X drug effect for each the low and high drug concentration, as well as baseline serotonin levels. Pair-wise comparisons of hSERT-wt mice in both drug groups were compared to determine if the same concentration of each SSRI resulted in the same response.

In the AUC analysis, Prism 6.0 (GraphPad) was used to plot raw serotonin concentrations over time. Prism then determined the AUC for the first two time points of drug exposure: samples ending at 80 and 100 minutes for the low concentration, and samples ending at 200 and 220 minutes for the high concentration. The AUC data were then analyzed as the percent change from baseline data: SPSS (IBM) was used to run a 2-way ANOVA to determine the genotype X drug effect for each the low and high drug concentration.

There were 8 mice in each of the 4 experimental groups (2 genotypes X 2 SSRIs). One hSERT-wt mouse in the fluoxetine group was excluded, resulting in only 7 mice in that group. Mice were 8-12 weeks old and only male mice were included in the microdialysis experiments.

The hypothesis in the microdialysis experiments was that both escitalopram and fluoxetine would produce similar increases in extracellular serotonin in the hippocampus, and then the serotonin levels would return to baseline. A second drug challenge would evoke a larger increase in extracellular serotonin. While there was no expected effect of genotype for the fluoxetine groups, the serotonin response to escitalopram was hypothesized to be smaller for the hSERT-Emory mice compared to the hSERT-wt mice.

Results

As previously described, the serotonin response to SSRI drug challenge was measured by comparing the peak response as a percentage of baseline levels, and as AUC of the response in the two dialysate samples after the initiation of SSRI challenge. These two methods will be discussed separately. First it is important to note that the SSRIs did evoke a significant increase in extracellular serotonin levels from baseline. The group means for the extracellular serotonin concentrations at baseline and peak response for the low and high concentration drug challenges can be seen in Table 2, all values are uncorrected for probe recovery. A repeated-measures ANOVA comparing the mean baseline, and peak response for both drug challenges found no genotype X dose X drug effect, $F(2,54) = 1.049$, $p = 0.357$. However, there was a significant main effect of drug challenge (dose), $F(2,54) = 8.187$, $p = 0.001$. Pairwise comparisons revealed that both the low and high SSRI challenges evoked a significant increase in extracellular serotonin, $p = 0.002$ and 0.006, respectively. There was no difference between the low and high concentrations, $p = 1.000$. There were no between subjects effects of genotype, $F(1,27) = 1.728$, $p = 0.200$. There were also no between subject effects of drug, $F(1,27) = 1.137$, $p = 0.296$.

Percent change from baseline values for each group can be seen in Table 3 and Figure 9. A 2-way ANOVA determined there was no genotype X drug effect for the low concentration challenge, $F(1,27) = 0.078$, $p = 0.783$. There was also no genotype X drug effect for the high concentration challenge, $F(1,27) = 0.465$, $p = 0.501$. There was also no genotype X drug effect for the high K+ challenge, $F(1,27) = 0.171$, $p = 0.682$. To confirm that all four groups had similar baselines and that was not affecting percent change from

baseline measurements, a 1-way ANOVA found baseline concentrations of serotonin are the same across the four experimental groups, $F(3,27) = 0.513$ $p = 0.677$ (Figure 10).

AUC values for each group can be seen in Table 4 and Figure 11. AUC was determined for the two samples that coincided with the 40 minutes after each drug challenge began. There was no genotype X drug effect at the low concentration of drug challenge, $F(1,27) = 1.528$, $p = 0.227$, however there was a modestly significant main effect of genotype, $F(1,27) = 4.231$, $p = 0.049$. There was no genotype X drug effect at the high concentration drug challenge, $F(1,27) = 0.761$, $p = 0.391$. There was no genotype X drug effect at the high K+ challenge, $F(1,27) = 0.712$, $p = 0.406$. There was no main effect of genotype for high concentration drug challenge or K+ challenge, $p =$ 0.684 and 0.512, respectively.

The low concentration drug challenge AUC was examined more in-depth due to the significant main effect of genotype. Student's t-test revealed there is no significant difference between the hSERT-wt mice in the escitalopram and fluoxetine groups, $p =$ 0.893. There was also no difference between the drug groups for the hSERT-Emory mice, $p = 0.224$. Within the escitalopram and fluoxetine groups individually, there was no significant effect based on genotypes, $p = 0.101$ and 0.122, respectively. While there is a modest main effect of genotype, it only a main effect and exists when the escitalopram and fluoxetine groups are combined. And because there is no interaction effect of genotype X drug, this modest effect is likely driven by the variation in the data than a true effect. Ultimately a genotype effect would only have value within a single drug group, so this main effect has no consequence in the overall interpretation of the data from the microdialysis experiment.

Conclusions

The microdialysis experiments were designed to measure the physiological role of escitalopram's allosteric site on the hSERT by evaluating alterations in serotonin efflux due to the presence or absence of the allosteric site. Previous work in rats indicated that the interaction of escitalopram and *R*-citalopram resulted in a blunted effect of escitalopram in serotonin efflux (Mørk et al., 2003). The previous experiments set the stage the hSERT-wt and hSERT-Emory mice, and now the experiments described here directly examined escitalopram's role at the allosteric site, without the interaction of *R*-citalopram. Initial analysis confirmed that in the knockin mice escitalopram and comparator fluoxetine do evoke the expected increase in extracellular serotonin. This was important to confirm because if the knockin mice were not responding to the SSRIs in the expected manner then the mice could not be a valid measure of the role of the allosteric site. It is also of note that hSERT-wt and hSERT-Emory mice do not have different basal levels of serotonin. It is reasonable that the absence of the allosteric site altered the activity of the hSERT in the mouse brain to result in different baseline serotonin. Had this been so, it would have required further examination of how serotonin levels changed after drug challenge.

While each the low and high concentrations of escitalopram and fluoxetine evoked an increase in extracellular serotonin in the ventral hippocampus, it is interesting that there was no difference between a 0.0462 μM concentration and 0.1 μM concentration for either drug. This is especially noteworthy because pilot experiments in heterozygous hSERT-wt/Emory mice clearly suggested the higher concentration would evoke a larger response for both escitalopram and fluoxetine. While the high

concentration drug exposure was only two hours after the low concentration exposure, there was a return to baseline serotonin level after the first exposure. Logically the transporter is no longer naïve to escitalopram or fluoxetine, but apparently the washout period was not enough to return the area around the microdialysis probe to a true baseline. Perhaps the serotonin increase after the first drug challenge resulted in activation of downstream serotonergic mechanism that left the local area in a refractory state where the system is unable to respond. Autoreceptors may have been activated that inhibit the release of serotonin. Perhaps the serotonin embargoed in the synapse was metabolized and the presynaptic cells did not replace the serotonin fully by the second drug challenge. Additionally, the effect of the lower concentration of drug may have been a ceiling effect, however the variation in the data as well as the pilot data suggests this is not the case. Each of these suppositions has merit, but the experiments were not setup to specifically explore them. Further study could include blocking autoreceptors or degradation of serotonin to address this, but those avenues of study would be about developing the microdialysis method rather than answering the question at hand about the role of escitalopram's allosteric site. A simple future direction to answer that question would be to give a larger concentration of drug than 0.1 μM. The lower and higher concentrations were only a third log-step apart; this may not have been enough separation despite the pilot data indicating that these were appropriate concentrations. Altogether, the lack of difference between the two doses prevents making a dose-response curve and determination of ED_{50} values with this data.

Once baseline serotonin levels were confirmed to be the same between the genotypes, the presence or absence of the allosteric site on the hSERT could be assessed

in how serotonin levels changed in response to drug challenge. Percent change from baseline examined if there was a change in peak response due to genotype. As expected, there was no genotype effect for the fluoxetine groups, but there was also no effect in the escitalopram group. This held true for both the low and high concentration of drug. This does not support the hypothesis that the allosteric site is altering serotonin efflux in the ventral hippocampus.

The AUC analysis examined if the response over time changed as a function of the allosteric site. The AUC analysis also matched the percent change over baseline results in there being no interaction of the genotypes and drugs for both concentrations of drug. As previously described, the data from the high concentrations of escitalopram and fluoxetine is may not be an accurate representation of the response to 0.1 µM escitalopram or fluoxetine, thus leaving a focus on the 0.0462 µM concentration. For the low concentration challenge there was a modest main effect of genotype. As pairwise comparisons within the individual drug groups reveals no significant effect of genotype on extracellular serotonin response. This indicates that for escitalopram there is no effect of the presence or absence of the allosteric site on serotonin efflux in the ventral hippocampus.

The results from this study are also in line with work completed with another line of knockin mice. Another group investigated the role of the allosteric site with a different allosteric-null hSERT. Their allosteric-null hSERT was composed of a different set of point mutations, which altered primary affinity of escitalopram unlike those for hSERT-Emory (Neubauer et al., 2006). Performing microdialysis in the frontal cortex, this group found no changes in serotonin efflux after an intravenous challenge of escitalopram

(Jacobsen et al., 2014). The group also noted that a sub-maximal dose of escitalopram did result in a genotype effect, they attributed this effect to the change in escitalopram's affinity at the primary site on the hSERT rather than to the absence of the allosteric site. These results couple with the hSERT-Emory studies to suggest that the allosteric site does not have a physiologically relevant role *in vivo* for escitalopram's mechanism of action.

Together the two methods of analysis on the hSERT-wt and hSERT-Emory mouse data are in agreement that at a physiological level in an *in vivo* system there is not an impactful role of the allosteric site in escitalopram activity at the hSERT. This finding is of course restricted to the ventral hippocampus and the serotonergic system there as serotonin efflux may be different in another region. At this time there is no data to suggest that microdialysis in another region would have reached a different conclusion, but only further experimentation in more brain regions would be conclusive

Figures

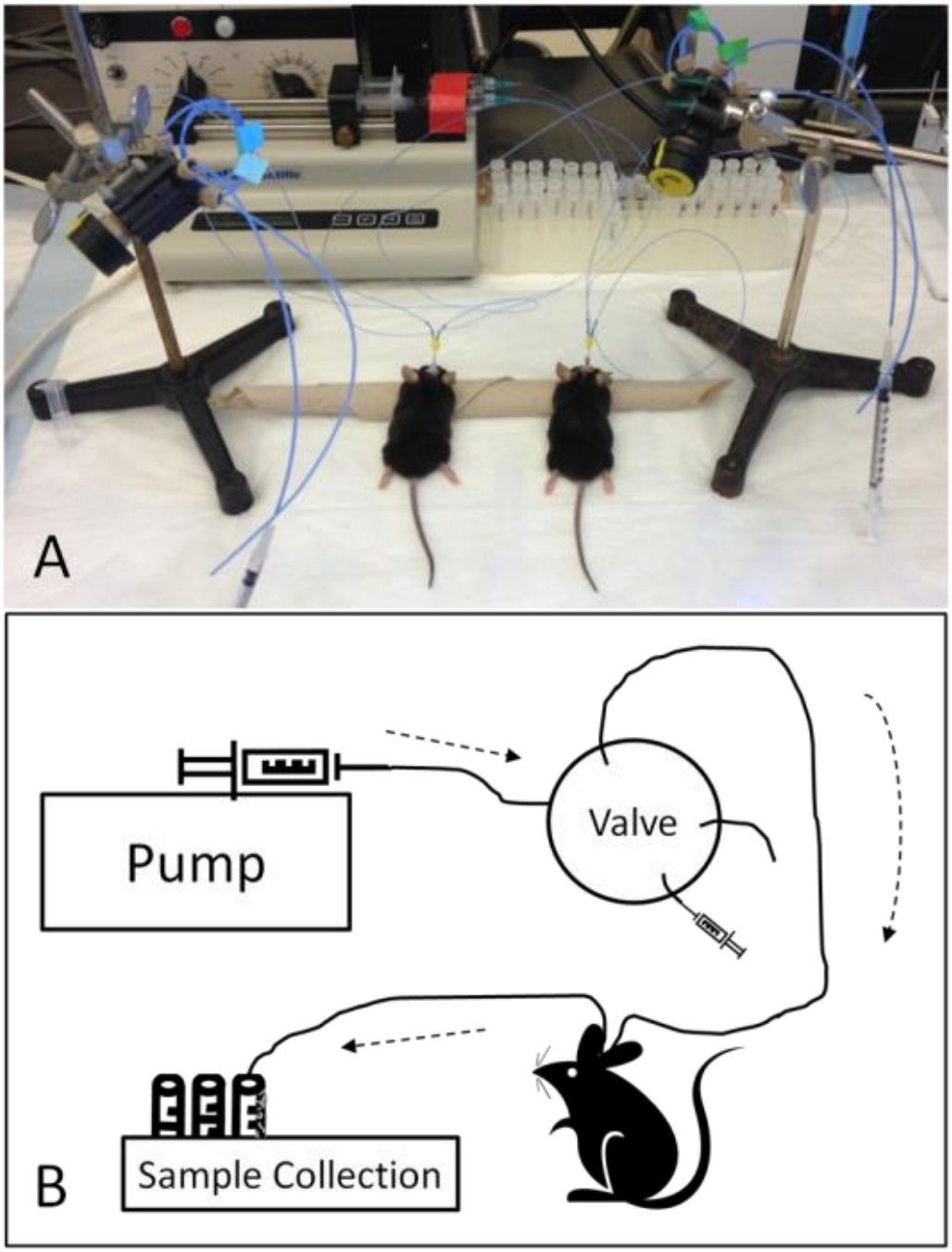

Figure 6: Schematic of microdialysis setup.

A. Picture of two mice during microdialysis. B. Cartoon of setup. Note aCSF flows from syringe in the pump, through the valve, to the probe in the mouse, then to sample collection tube.

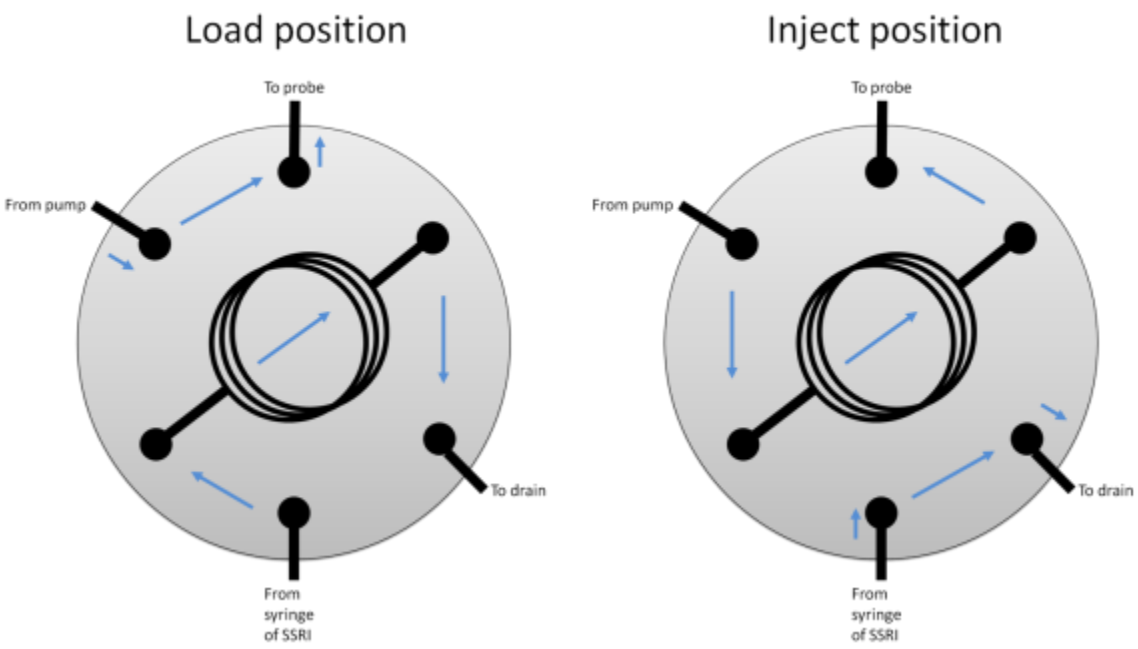

Figure 7: Schematic of 6-port valve.

In the load position, aCSF from the pump flows directly to the probe, bypassing the loop, while the syringe of SSRI is able to fill the loop. In the inject position, aCSF from the pump flows through the loop, pushing the solution in the loop to the probe, while the syringe of SSRI flows directly to the waste drain.

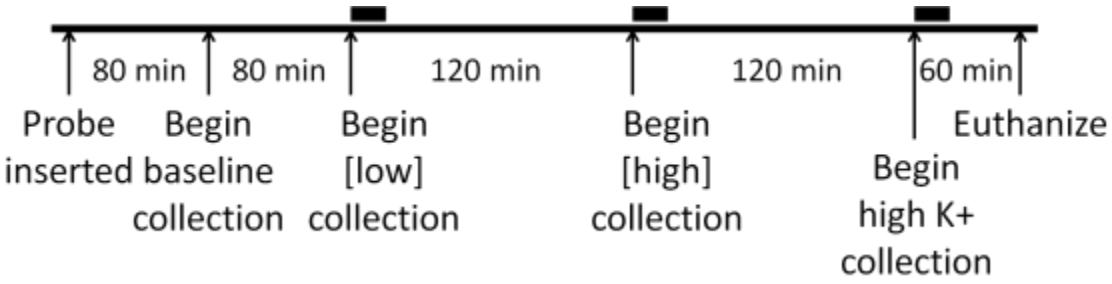

Figure 8: Microdialysis timeline.

Sample collected every 20 minutes. Time = 0 min at when baseline collection begins. The thick bar above the timeline indicates time of drug exposure. Probe was removed from the mouse at time of euthanasia. Serotonin recovery was performed before and after the probe was in the mouse. The time of drug exposure matched with the first sample for the drug challenge.

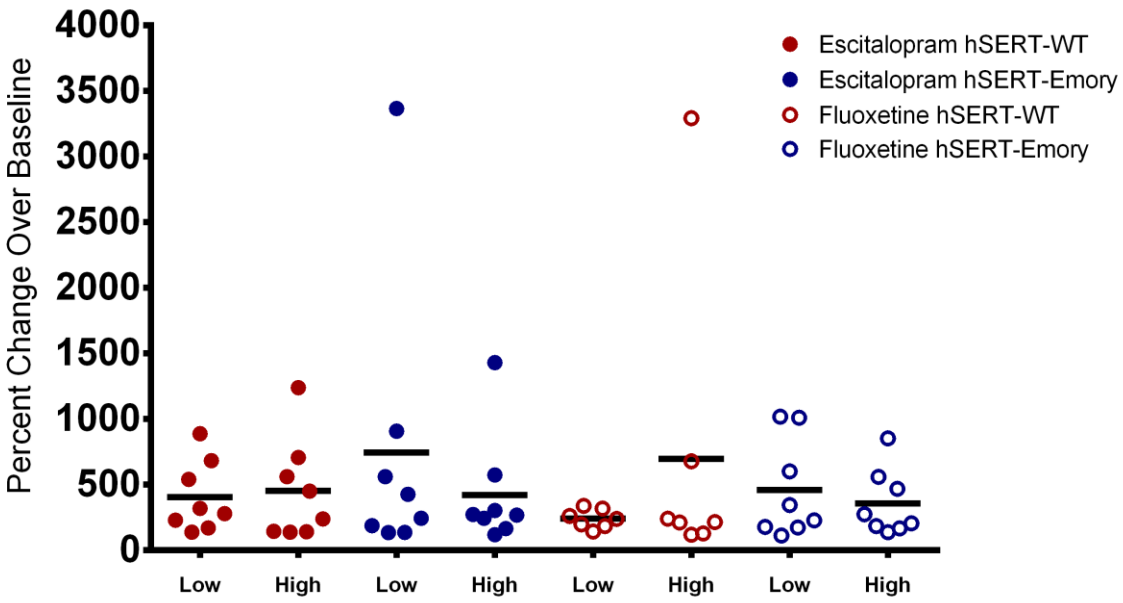

Figure 9: Percent change from baseline of extracellular serotonin.

Scatter plot with mean bar. Red: hSERT-wt; Blue: hSERT-Emory; Filled circles: escitalopram; Open circles: fluoxetine.

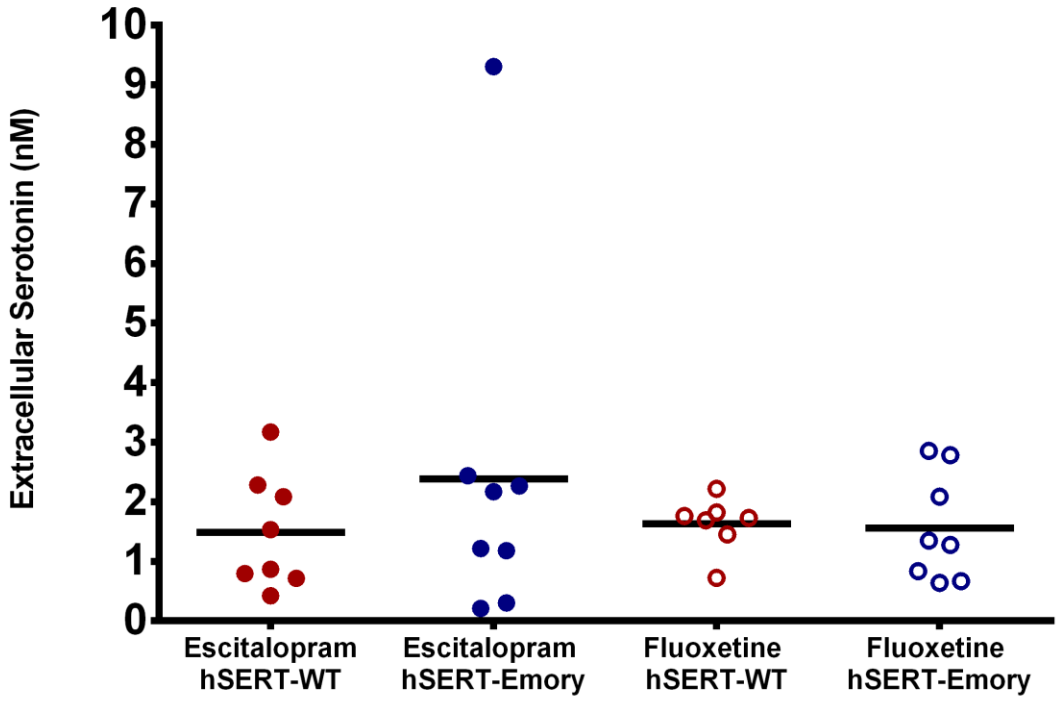

Figure 10: Baseline extracellular serotonin levels.

Raw values uncorrected for probe recovery. Red: hSERT-wt; Blue: hSERT-Emory; Filled circles: escitalopram; Open circles: fluoxetine. No significant differences between groups, $F(1,27) = 0.513$, $p = 0.677$.

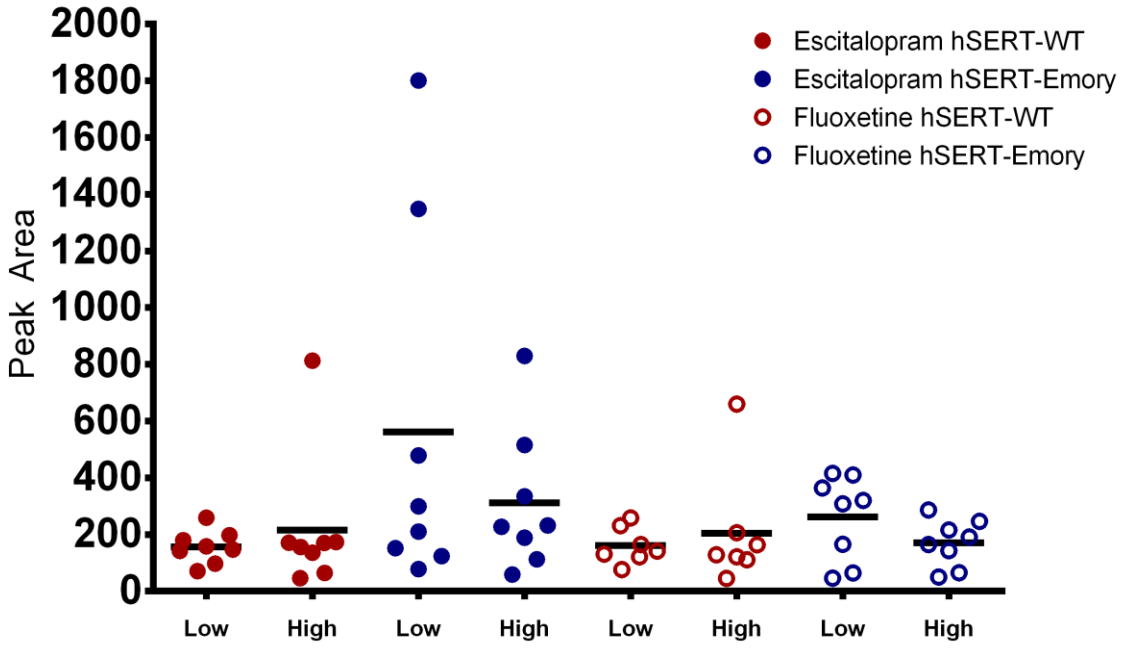

Figure 11: Area under the curve of extracellular serotonin.

Drug exposure coincided with the first 20 minutes and is the 40 minutes after drug exposure. Scatter plot with mean bar. Red: hSERT-wt; Blue: hSERT-Emory; Filled circles: escitalopram; Open circles: fluoxetine. There is a significant main effect of genotype for the low concentration of drug challenge, $F(1,27) = 4.231$, $p = 0.049$.

Tables

serotonin concentration (nM)	Escitalopram		Fluoxetine	
	hSERT-wt (n=8)	hSERT-Emory (n=8)	hSERT-wt (n=7)	hSERT-Emory (n=8)
Baseline	1.5 ± 0.34	2.4 ± 1.0	1.6 ± 0.17	1.6 ± 0.32
Low	3.5 ± 0.31	14.4 ± 5.45	3.8 ± 0.62	6.1 ± 1.5
High	5.4 ± 2.5	6.8 ± 1.8	6.9 ± 3.9	3.8 ± 0.67

Table 2: Serotonin concentrations.

All values uncorrected for probe recovery. Baseline is mean of 4 baseline values, low and high are the peak response after drug challenge. No significant effect of genotype X dose X drug, $F(2,54) = 1.049$, $p = 0.357$. However, there was a significant main effect of dose, $F(2,54) = 8.187$, $p = 0.001$, with both drug challenges causing a significant increase in serotonin, $p < 0.05$. No significant difference between low and high concentration responses. Mean ± SEM.

% change from baseline	Escitalopram		Fluoxetine	
	hSERT-wt (n=8)	hSERT-Emory (n=8)	hSERT-wt (n=7)	hSERT-Emory (n=8)
Low	406.5 ± 95.30	746.0 ± 385.8	240.5 ± 27.07	458.9 ± 132.4
High	453.0 ± 135.8	422.6 ± 151.7	697.9 ± 438.3	356.6 ± 88.97
High K+	1041.8 ± 236.2	1017.7 ± 185.7	706.7 ± 178.6	839.5 ± 139.8

Table 3: Percent change from baseline.

No significant differences between the experimental groups for low or high SSRI concentration or high K+ challenges. Mean ± SEM.

AUC (nM by time)	Escitalopram		Fluoxetine	
	hSERT-wt (n=8)	hSERT-Emory (n=8)	hSERT-wt (n=7)	hSERT-Emory (n=8)
Low	156.5 ± 20.67	561.3 ± 229.4	160.8 ± 24.15	261.7 ± 52.77
High	216.3 ± 87.07	312.4 ± 88.87	205.0 ± 78.02	170.5 ± 29.29
High K+	500.3 ± 206.9	787.1 ± 293.7	340.8 ± 57.60	306.9 ± 54.78

Table 4: Area under the curve.

No significant differences between the experimental groups for low or high SSRI concentration or high K+ challenges. There is a significant main effect of genotype for the low drug challenge, $F(1,27) = 4.231$, $p = 0.049$. Mean ± SEM.

Chapter 5: Behavior

Abstract

The marble burying (MB) and tail suspension (TST) tests were used to measure the behavioral output of escitalopram activity and if the presence or absence of the allosteric site on the hSERT altered the behavioral effects. There was a significant effect of genotype in the MB, in which a larger dose of escitalopram is required for hSERT-Emory mice to see the same reduction in marbles buried as for hSERT-wt mice. This effect was not due to a locomotor effect by escitalopram. There was no significant effect of genotype in the TST; increased escitalopram dose reduce duration immobile similarly for both hSERT-wt and hSERT-Emory mice. The incongruence of the two behavioral measures is likely explained by MB being more sensitive to fine changes in extracellular serotonin caused by the presence of the allosteric site, while the TST is not sensitive to these fine changes.

Introduction

An essential part to understanding the role of escitalopram's allosteric site on the hSERT is understanding its behavioral role. Any changes in biochemistry and physiology are only truly valuable with accompanying behavioral changes. Behavior is the metric to observe global effects of the allosteric site in escitalopram's mechanism of action. In a patient prescribed escitalopram, the biochemical and physiological changes happening to the serotonergic system only have meaning if psychiatric symptoms improve, which is the behavioral output. Between patients, symptom relief can be quite variable, a pattern common not only to escitalopram but to SSRIs in general (Healy, 2000; Möller, 2000). While biochemical and physiological analyses bear knowledge about the mechanistic role of the allosteric site, behavioral assays are paramount in understanding if the biochemical and physiological effects lead to global improvement of symptoms.

Antidepressants, including SSRIs, have a whole battery of behavioral assays for assessment in mice (Dekeyne, 2005; Deussing, 2006; Yan, Cao, Das, Zhu, & Gao, 2010). To study the hSERT-wt and hSERT-Emory mice tests were sought that could reliably and accurately screen escitalopram's behavioral effects in the presence or absence of the allosteric site. Pilot testing determined that both the marble burying test (MB) and the tail suspension test (TST) would be appropriate tests. Having two behavioral tests also provides a more complete view of how the allosteric site is involved in escitalopram-induced behaviors.

MB is a simple task that utilizes a mouse's natural digging and burying behaviors. Mice will bury both noxious and innocuous items, including glass marbles (Njung'e & Handley, 1991b). SSRIs are known to reduce burying behavior (Njung'e & Handley,

1991a). There are several proposals suggesting what MB models, with suggestions including measure of anxiety or compulsiveness (Njung'e & Handley, 1991b; Thomas et al., 2009). These hypotheses are bolstered by the use of SSRIs to treat anxiety and obsessive-compulsive disorders. While this face validity can help interpret the results of the behavioral assay, here MB was specifically used as a known screen of SERT antagonism, which is the high predictive validity of the assay. The hSERT-wt and hSERT-Emory mice are not modeling any psychiatric disorder, rather are tools for *in vivo* examination of the allosteric site. In addition to being an SSRI screen, MB is replicable within subjects and robust on consecutive days of testing (Thomas et al., 2009). These qualities makes it possible for an individual animal to complete a full range of experimental drug doses and consequently make a more accurate dose-response curve for escitalopram in each knockin mouse line. Altogether the MB assay is a reliable and accurate test to examine the allosteric site.

Like MB, TST is a well-developed assay in mice. This often used assay is thought to be a useful model for depression, and more importantly it is responsive to SSRIs and used as a screen of SSRIs (Ripoll et al., 2003; Steru, Chermat, Thierry, & Simon, 1985). SSRIs, including escitalopram, are able to decrease time spent immobile during the test (Crowley, Blendy, & Lucki, 2005; Crowley, Brodkin, Blendy, Berrettini, & Lucki, 2006; Mombereau, Gur, Onksen, & Blendy, 2010). Pilot work, to be discussed in following sections, determined that like MB, the assay is repeatable within subjects, thus making TST a robust tool.

MB and TST are hypothesized to model different aspects of psychiatric disorders. MB is thought to model the anxiety-like phenotypes of disorders SSRIs are used to treat

while TST is thought to model the depressive-like phenotypes of disorders SSRIs are also used to treat. What links them is that SSRIs, including escitalopram, are used to treat the variety of disorders that the two behavioral assays may model, and thus these tests can be used as screens of SSRI activity. Again, what these tests model is up for some debate and may only be endophenotypes of what are complex human disorders. Together, though, MB and TST can provide a more complete look at how the allosteric site plays a role in escitalopram action. The assays are not meant to model the same, or necessarily any specific, human behavior, but they are meant to study the same class of human drugs. The hSERT-wt and hSERT-Emory mice are not models of psychiatric disorders rather a set of tools to examine escitalopram. Paired together the two assays provide a more complete measure of the range of behaviors escitalopram affects, and in turn the range of behaviors that may be altered by the presence or absence of the allosteric site on the hSERT.

Marble Burying

Materials and Methods

The MB assay can be performed a number of ways, all with the same general pattern of introducing a number of items that an animal can bury during a set period of time. Black marbles with a diameter of 1 centimeter are ideal for mice because they are of a size that the mouse is capable of burying but not too small that a mouse could step on it and accidentally bury a marble. Here, the MB session used 20 marbles arranged in a 4 by 5 grid (Figure 12). The arena was a clean rat cage, 42 centimeters long by 22 centimeters wide by 20 centimeters tall. Corncob bedding same as the home cage bedding was used to make a 4 centimeter thick layer on the bottom of the arena. This bedding was familiar to the mice and thick enough to bury the marbles. The individual MB session

lasted 60 minutes, during which the mouse was allowed free movement within the MB arena, but had no access to food or water. After 60 minutes the mouse was promptly returned to its group housing home cage, resuming *ad libitum* access to food and water. Lighting was similar to typical levels during the mouse's light cycle, and all testing was performed during the mouse's light cycle.

Pilot testing was completed to determine appropriate drug dosing levels and that the assay was repeatable within subject. Previous reports detailed that MB is repeatable, but it was important to confirm that held true for the specifications of this assay and with SSRI use. It was also necessary to confirm that escitalopram evoked an effect in MB, and that fluoxetine was an appropriate comparator SSRI. Pilot results will be described below, but in short it was determined that escitalopram evokes an effect in MB, and fluoxetine is an appropriate comparator SSRI. Fluoxetine was chosen as the comparator SSRI because it has no allosteric activity. The assay was repeatable over the necessary 9 weeks to complete the desired full dose-response curve for both drugs.

The final assay paradigm included an individual mouse completing 8 MB sessions, once per week. The first 4 sessions would with either escitalopram or fluoxetine, followed by a week of rest, then 4 weeks of the other SSRI (Figure 13). The drug order was randomized across the experimental cohort so that each week animals were in each of the four drug doses for both drugs. Escitalopram, as escitalopram oxalate (gift from Lundbeck A/S), was dissolved in hospital grade 0.9% saline, to doses of 0, 1, 3.2, and 10 mg/kg (mg of the salt per kg of body weight), and administered in 200 µL volumes per 30 g mouse weight. Fluoxetine, as fluoxetine HCl (Sigma-Aldrich), was also dissolved in saline, to doses of 0, 1, 10, and 32 mg/kg (mg of the salt per kg of body

weight), and administered in 200 µL volumes per 30 g mouse weight. Each MB day the mouse was weighed for accurate dosing. Thirty minutes prior to the MB session the mouse would receive an intraperitoneal injection (i.p.) of the drug and dose for that session, and be returned to home cage. After 30 minutes the mouse would be placed in the MB arena for the 60 minute session. Every 15 minutes the mice would be observed and buried marbles counted. Although 4 counts were made over the course of a MB session, only the final count of marbles buried was analyzed. A marbled was buried if more than half of it was buried. Pilot studies were filmed and later analyzed for locomotion (described in a following section). Studies with the hSERT knockin mice were not filmed.

The pilot studies were completed in wildtype C57BL/6J mice (Jackson Lab). Only males were used, and they were 8-10 weeks old at the beginning of the experiment. The experimental studies included male and female hSERT-wt, hSERT-Emory, and hSERT-wt/Emory mice, 8-12 weeks old at the start of the experiment.

Statistics were carried out using SPSS (IBM). A repeated-measures ANOVA was used to examine genotype X dose X drug effects for the knockin mice. Pilot studies were analyzed using a 1-way ANOVA to identify a drug effect. ED_{50} values were determined and compared in Prism 6.0 (GraphPad).

Results

Pilot work identified a significant effect of drug for escitalopram, $F(3,15) = 10.359$, $p = 0.001$ (Figure 14). Post hoc tests revealed that doses of 3.2 and 10 mg/kg escitalopram oxalate resulted in a decrease in buried marbles compared to saline, $p < 0.05$ for both. Additionally there was a drug effect for fluoxetine, $F(3,15) = 6.275$, $p = 0.006$

(Figure 15). Post hoc tests revealed that a dose of 32 mg/kg fluoxetine HCl resulted in a decrease in buried marbles compared to saline, $p < 0.05$. These data confirmed the feasibility and validity of the test for use in the knockin mouse lines.

For the experiments with the knockin mice, mice were removed if they buried 0-2 or 18-20 marbles in the saline condition for either drug. These mice were two standard deviations from the mean number of marbles buried without drug on board. Additionally, pilot testing indicated that increasing doses of escitalopram and fluoxetine decreased the number of marbles buried per MB session. Mice that buried 0-2 marbles had no latitude to bury fewer marbles and mice that buried 18-20 marbles could only bury fewer marbles. Additionally, any mouse that did not complete all 8 MB sessions was excluded.

There was variation in saline burying, thus the knockin mouse data were normalized. The raw values of marbles buried per drug dose were normalized to the individual mouse's number of marbles buried in the saline condition. Because each mouse had two saline values, one per each block of four weeks of an individual drug, the number of marbles per drug was normalized to the saline week that matched. For example, if a mouse received escitalopram in the first four weeks then fluoxetine in the second four weeks, the week 1-4 saline value was used to normalize the escitalopram weeks while the week 5-8 saline value was used to normalize the fluoxetine weeks. This normalized data was then curve fit for analysis and determination of ED_{50} values. The bottom of the curve was constrained to 0 because a mouse could not bury fewer than 0 marbles. The top of the curve was constrained to 1 because a mouse could not bury more than 100% of the marbles.

In the knockin lines, there was a significant genotype X dose X drug effect, $F(6,186) = 2.516$, $p = 0.023$ (Figure 16, Figure 17). There was a significant main effect of dose, $F(3,186) = 67.676$, $p < 0.001$, indicative that the MB test in the knockin mice followed the expected effect based on the pilot test. The escitalopram oxalate ED_{50} for hSERT-wt was 2.528 mg/kg, hSERT-Emory was 24.06 mg/kg, and hSERT-wt/Emory was 14.41 mg/kg (Figure 16 inset). There was a significant effect of genotype on the escitalopram ED_{50} values, $F(2,257) = 3.175$, $p = 0.0435$. Post hoc tests revealed there was a significant difference between hSERT-wt and hSERT-Emory ED_{50}, $p < 0.05$. The fluoxetine HCl ED_{50} for hSERT-wt was 6.450 mg/kg, hSERT-Emory was 12.23 mg/kg, and hSERT-wt/Emory was 8.598 mg/kg (Figure 17 inset). There was no significant effect of genotype on the fluoxetine ED_{50} values, $F(2,257) = 1.381$, $p = 0.2533$.

These results from the knockin mice support the hypothesis that the allosteric site for escitalopram on the hSERT does have a behavioral role in the MB. The significant increase in ED_{50} in the absence of the allosteric site on the hSERT indicates a rightward shift in the dose-response curve for escitalopram. Consequently more escitalopram is necessary to achieve the same effect on the MB test in hSERT-Emory mice compared to intact hSERT-wt mice. No significant change in ED_{50} for allosteric-inactive fluoxetine between genotypes further bolsters the hypothesis that the observed escitalopram effect is due to the presence or absence of the allosteric site.

Locomotion

Materials and Methods

To rule out the possibility that the effects observed in the MB assay were due to a locomotor effect of the SSRIs, locomotion was measured in the pilot MB assays with

wildtype C57BL/6J. Total distance travelled in the MB arena was measured using Clever Sys Software (Clever Sys Inc.). Film of the pilot MB assay was used for this analysis. Individually for each drug, SPSS was used to perform a 1-way ANOVA, significance set at $p < 0.05$.

Results

There was no significant effect of escitalopram dose on distance travelled in wildtype mice, $F(1.723, 12.061) = 3.230$, $p > 0.05$ (Figure 18). There was a significant effect of fluoxetine dose in wildtype mice, $F(1.964, 21.599) = 25.602$, $p < 0.001$. Post hoc tests revealed a fluoxetine HCl dose of 32 mg/kg decreased the distance travelled compared to all other doses, $p < 0.05$ (Figure 19).

The locomotor measurements were meant to aid in interpreting the marble burying results. The lack of effect of escitalopram on distance travelled indicates that decreased marble burying at higher escitalopram doses is not due to the mouse not moving that precludes burying, rather a lack of burying alone. The highest dose of fluoxetine did have a significant decrease in distance travelled, suggesting this could be at play in the observed dose effects of fluoxetine on MB, but this is only influences the interpretation of genotype effect in MB. As that there was no genotype effect for fluoxetine in MB, the decrease in locomotion at higher doses is not of concern.

Tail Suspension Test

Materials and Methods

The TST is a standard screen of SSRI action, making it an optimal screen of the allosteric site for escitalopram on the hSERT. Mice were affixed to a horizontal bar with tape 30 cm from the ground (Figure 20). A piece of plastic 1 cm in length was put on the

tail of each mouse to prevent tail climbing. Each TST session lasted 6 minutes and was filmed for later scoring. Within the 6 minute TST session duration immobile was scored using Stopwatch+ (Center for Behavioral Neuroscience, Emory University). Immobility was defined as no movement of limbs or head. Stopwatch+ also recorded latency to first immobility event. After the TST session the mouse was promptly returned to its group housing home cage, resuming *ad libitum* access to food and water. Lighting was similar to typical levels during the mouse's light cycle, and all testing was performed during the mouse's light cycle.

Pilot testing was completed to determine escitalopram dosing and appropriate allosteric-inactive SSRI comparator drug. Pilot testing also identified if escitalopram evoked a response in mice and if the TST was repeatable within subjects. In short, the TST is repeatable and escitalopram has an effect on the test. Fluoxetine was not a suitable comparator SSRI, but allosteric-inactive fluvoxamine proved to be a suitable control. Full results are discussed in the following sections. Unlike the experimental design described below, for pilot escitalopram and fluoxetine testing, mice were in either the saline group and repeated multiple weeks at the saline dose, or the drug group, completing multiple drug concentrations. Fluvoxamine pilot studies were completed as below.

The final TST assay followed a similar timeline to the MB assay. Eight TST sessions were completed over 9 weeks, with each mouse completing 4 escitalopram oxalate doses (0-10 mg/kg) and 4 fluvoxamine maleate doses (0-32 mg/kg) in four weeks (Figure 21). A 2 week washout period occurred between compounds and the order of the drugs and doses was counterbalanced across the testing cohort. Escitalopram, as escitalopram oxalate (gift from Lundbeck A/S), was dissolved in hospital grade 0.9 %

saline, to doses of 0, 1, 3.2, and 10 mg/kg (mg of the salt per kg of body weight), and administered in 200 µL volumes per 30 g mouse weight. Fluvoxamine, as fluvoxamine maleate (Sigma-Aldrich), was also dissolved in saline, to doses of 0, 1, 10, and 32 mg/kg (mg of the salt per kg of body weight), and administered in 200 µL volumes per 30 g mouse weight. Each TST day the mouse was weighed for accurate dosing. Thirty minutes prior to the TST session the mouse would receive an intraperitoneal injection (i.p.) of the drug and dose for that session, and be returned to home cage. After 30 minutes the mouse would be placed for the TST for the 6 minute session. Sessions were scored without knowledge of genotype, drug, or dose.

For the pilot tests wildtype C57BL/6J mice (Jackson Lab) were used. The mice were male and 8-10 weeks old at the start of the experiment. In the experiments with the knockin mice, male and female hSERT-wt, hSERT-Emory and hSERT-wt/Emory mice, aged 8-12 weeks at the beginning of the experiment.

Statistics were carried out using SPSS (IBM) as with MB. A repeated-measures ANOVA was used to examine genotype X dose X drug effects for the knockin mice. Pilot studies were analyzed using a 1-way ANOVA to identify a drug effect. ED_{50} values were determined and compared in Prism 6.0 (GraphPad).

Results

Pilot testing with escitalopram found a significant effect of dose in duration immobile in the TST, $F(3,45) = 22.2$, $p < 0.001$ (Figure 22). Post hoc tests revealed a significant decrease in duration immobile at the 10 mg/kg escitalopram oxalate dose, $p < 0.05$. Fluoxetine, as fluoxetine HCl (Sigma-Aldrich), was initially examined as the comparator SSRI, but a suitable dose effect was observed in the wildtype mice, $F(3,45) =$

10.0, $p < 0.001$, as that the lowest measured fluoxetine HCl dose, 10 mg/kg, was significantly different from the saline dose ($p < 0.05$), but the higher doses were not significantly different (Figure 23). Further, in the pilot study the drug order was not counterbalanced and all mice in the 10 mg/kg fluoxetine HCl group had their TST session the same day. On that day there was no significant difference between the 10 mg/kg fluoxetine HCl group from the saline group ($p > 0.05$). Consequently fluvoxamine was piloted as the comparator SSRI in the TST experiment. Fluvoxamine has a similar pattern of effect in the TST as escitalopram, with a significant effect of dose in duration immobile in the TST, $F(3,93) = 20.1$, $p < 0.001$ (Figure 24). Post hoc tests revealed a significant decrease in duration immobile at the 32 maleate fluvoxamine dose, $p < 0.05$.

Mice that did not complete all 8 TST sessions were excluded from data analysis in the knockin mouse studies. Any mouse that was immobile for fewer than 60 seconds or longer than 300 seconds of the 6 minute session was also excluded from analysis. Pilot work did suggest repeated TST sessions would result in some learning of the task, and while counterbalancing was meant to negate that effect, analysis did determine there was no difference in the saline group mice week to week, $F(7,51) = 1.806$, $p = 0.110$. This suggested that while learning may have occurred, the counterbalancing the order of dose negated its potential effect.

There was variation in the saline doses and across the drug doses in the TST data, so the data was normalized. The raw values of duration immobile per drug dose were plotted and fit to a curve. The unconstrained value for the top of this raw data fit curve was then used to normalize the raw data. This was done individually by genotype. The normalized data was then fit to a curve for further data analysis and determination of

ED_{50} values. For curve fitting the curve was constrained at the bottom to 0 because the mouse could not be immobile for less than 0 seconds. The top of the curve was constrained to 1 because a mouse could not be immobile more than 6 minutes.

In the knockin lines there was no significant genotype X dose X drug effect in duration immobile in the TST, $F(2.674, 44) = 0.454$, $p = 0.694$ (Figure 25, Figure 26). There was a significant main effect of dose in duration immobile in the TST, $F(1.308, 57.547) = 41.149$, $p < 0.001$, indicative that the TST worked as expected from pilot testing. The ED_{50} values were also examined for a genotype effect. The escitalopram oxalate ED_{50} for hSERT-wt was 4.770 mg/kg, hSERT-Emory was 8.205 mg/kg, and hSERT-wt/Emory was 5.883 mg/kg (Figure 25 insert). There was no significant effect on genotype for the escitalopram oxalate ED_{50} values, $F(2,182) = 0.7307$, $p = 0.4830$. The fluvoxamine maleate ED_{50} for hSERT-wt was 10.11 mg/kg, hSERT-Emory was 16.57 mg/kg, and hSERT-wt/Emory was 13.16 mg/kg (Figure 26 inset). There was no significant effect of genotype on the fluvoxamine maleate ED_{50} values, $F(2,182) = 0.6931$, $p = 0.5014$.

Like for duration immobile in the TST, there was no significant effect of genotype X dose X drug for latency to first immobility event in the TST, $F(2.674,58.837) = 0.454$, $p = 0.694$ (Figure 27, Figure 28). There was a main effect of dose on latency in the TST, $F(1.308,57.547) = 41.149$, $p < 0.001$. Post hoc tests revealed that there was a significant increase in latency in the TST for both escitalopram and fluvoxamine at their respective two highest doses.

These data suggest that escitalopram's allosteric site on the hSERT has no behavioral role in the TST. There was no genotype effect on either duration immobile or

latency to immobility, and there was no genotype effect on ED_{50} for escitalopram in the TST. This does not support the hypothesis that the absence allosteric site would cause a shift in behavioral output in the TST. The main effect of dose for both duration and latency does indicate that the TST was a successful test as suggested by the pilot work, however the presence or absence of the allosteric site does not alter the escitalopram effect in the TST.

Conclusions

Behavioral measures of the role of escitalopram's allosteric site on the hSERT are essential in showing that the *in vitro* effects of the site have a functional *in vivo* effect. Without the *in vivo* effect, the allosteric site is just an interesting but useless piece of pharmacology. The hypothesis is that the absence of the allosteric site will result in a need for a greater amount of escitalopram to achieve the same effect. This hypothesis was supported by the MB data but not the TST data. At first pass these results seem incongruous, but there are several reasonable explanations for these results. First, one of these results could be not real. Second, differences in the role of the serotonergic system in the MB and the TST could underlie the different results. Both of these explanations have merits, which will be discussed below.

The supposition that the results for the MB or TST test may not be representative of the true role of the allosteric site in these behavioral tests is possible. The genotype effect on ED_{50} values in the MB is modest, $p = 0.0435$, just under the set $p = 0.05$ threshold. It is possible that a few outliers could have driven the data. However, the fit curves of the dose-response MB data for each genotype do separate for escitalopram, whereas the fit curves for the genotypes overlap in the fluoxetine data for MB and both

SSRIs in the TST data. Additionally, the robustness of the significant effect of dose in the pilot and experimental data for both MB and TST indicate that there is a true dosing effect that is maintained. Altogether, it seems unlikely that a statistical anomaly was found; rather the escitalopram genotype effect in the MB is real, if modest. The number of mice in each experimental group was at least 10, enough for reasonable statistical analysis. There was substantial spread in the data, resulting in the normalization, but that is to be expected in behavioral data. Overall it is reasonable to conclude that there is no genotype effect in the TST but there is in MB.

After concluding that the observed effects are true, this leads to the hypothesis that there is a difference in the role of the serotonergic system in the MB and the TST. In the hypothesis for what the allosteric site's role is on the hSERT is the idea that escitalopram binding to the allosteric site results in escitalopram bound in the primary site to stay bound longer. The longer escitalopram is bound in the primary site the longer the hSERT is inhibited, allowing for greater accumulation of extracellular serotonin (Figure 1). Logically this serotonin is not patiently queuing until it may enter the cell, like molecular concertgoers at a theater door. This serotonin may queue, but it may also continue to act in the synapse, presumably resulting in the downstream effects of SSRIs. The delayed end to escitalopram's inhibition of the hSERT is not expected to be lengthy, thus only resulting in a modest local increase of extracellular serotonin compared to inhibition without the effects of the allosteric site. This would only be a fine tuning. It is possible that at some synapses that a fine tuning of serotonin levels would result in a downstream effect on postsynaptic cells, or even presynaptically. At other synapses the

primary inhibition by a SSRI may saturate the potential response, and a fine change makes no difference.

This fine tuning effect could carry through to the behavior data. The proposed fine tuning of escitalopram via its allosteric site evokes an effect in the MB but not in the TST. Anatomical specificity could then underlie this effect. As a classic test of antidepressants' activity, more is known about the neurochemical mechanisms of the TST. Norepinephrine, a system that interacts with the serotonin system, is known to play a role in the TST. For example, knocking out dopamine-β-hydroxylase, which converts dopamine to norepinephrine, disrupts the antidepressant effect of SSRIs, implicating the need for norepinephrine in for SSRI activity (Cryan, Mombereau, & Vassout, 2005). Mouse knockouts of either 5-HT_{1B} or 5-HT_{2C} receptors results in increased sensitivity to antidepressants, suggesting that these serotonin receptors play a role in the downstream effects of SSRIs (Cryan et al., 2005). Knockouts of the 5-HT_{1A} receptor has an antidepressant effect while eliminating the effect of SSRIs in the TST (Cryan et al., 2005). While less is known for MB, inhibiting the 5-HT_{1A} receptor using an antagonist also results in a blockade of the SSRI effect in MB (Ichimaru et al., 1995). Inhibiting norepinephrine uptake results in an antidepressant-like effect in both TST and MB (Cryan et al., 2005; Sugimoto, Tagawa, Kobayashi, Hotta, & Yamada, 2007). The interplay of serotonin and norepinephrine systems is not surprising, and it is possible that is how serotonin levels are affected by the presence or absence of the allosteric site. Possibly a fine difference in serotonin levels acting on norephinephrine underlies the separation in escitalopram's genotype effect in MB from TST.

Functionally it is also reasonable that regional specificity of serotonergic action could underlie the MB and the TST effects. The motor actions of burying and struggling while suspended are presumably different. It is reasonable that the neural systems of burying are sensitive to small changes in serotonin whereas those of struggling are not. The best future direction to support the hypothesis that fine changes in serotonin levels between mice with and without the allosteric site result in changes in escitalopram effects in MB but not the TST would be mapping the anatomical pathways of the two tasks. Then those pathways could systematically be analyzed for their role.

Overall it seems most likely that the observed MB and TST effects are true effects. The different role of the allosteric site in escitalopram's activity in these behavioral tests can be explained by different sensitivities to fine changes in extracellular serotonin levels. The exact locus of these varying sensitivities is unexplained as of now, but future understanding of the neural mechanisms of these tasks may provide explanation.

Figures

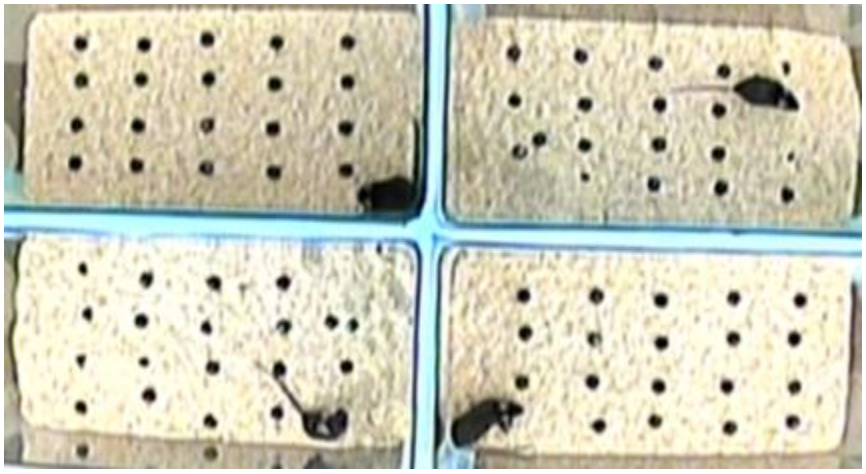

Figure 12: Arial view of marble burying arena.

Twenty black marbles (1 cm diameter) evenly arranged in a 4 by 5 grid. Note mice in the upper right and lower left arenas have moved and buried some of the marbles. Arena is 44 X 22 X 20 cm.

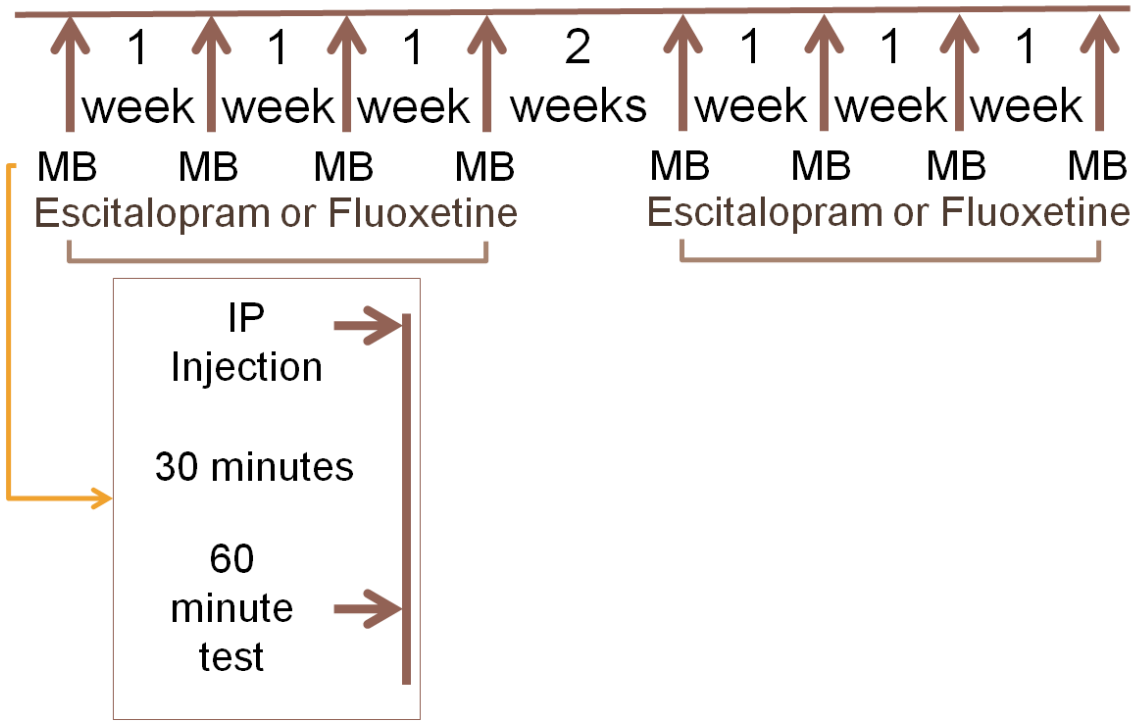

Figure 13: Marble burying schematic.

Each mouse had 8 MB sessions over 9 weeks. Each mouse had the 4 doses of escitalopram oxalate (0-10 mg/kg) or 4 doses of fluoxetine HCl (0-32 mg/kg) over the first 4 weeks, followed by 2 week washout period, then 4 weeks using the other SSRI. Per individual session the drug was administered 30 minutes prior to the test via i.p. injection.

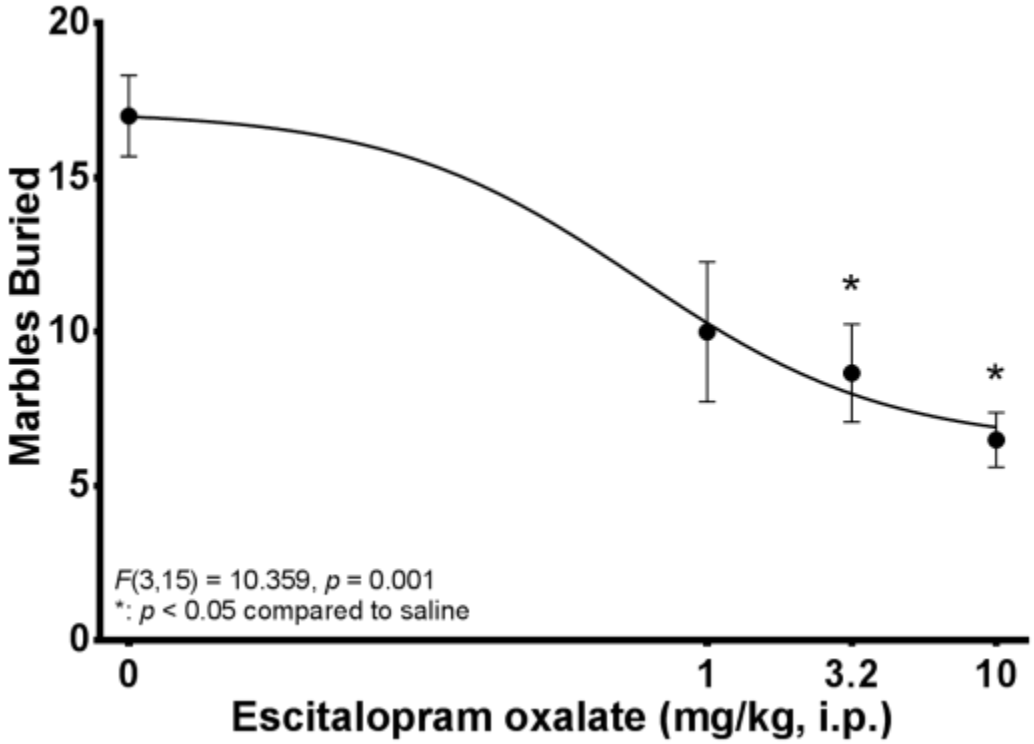

Figure 14: Pilot test of marble burying with escitalopram oxalate.

N=8 wildtype C57BL/6J mice. Significant effect of drug with decreased number of marbles buried at 3.2 and 10 mg/kg. Mean ± SEM.

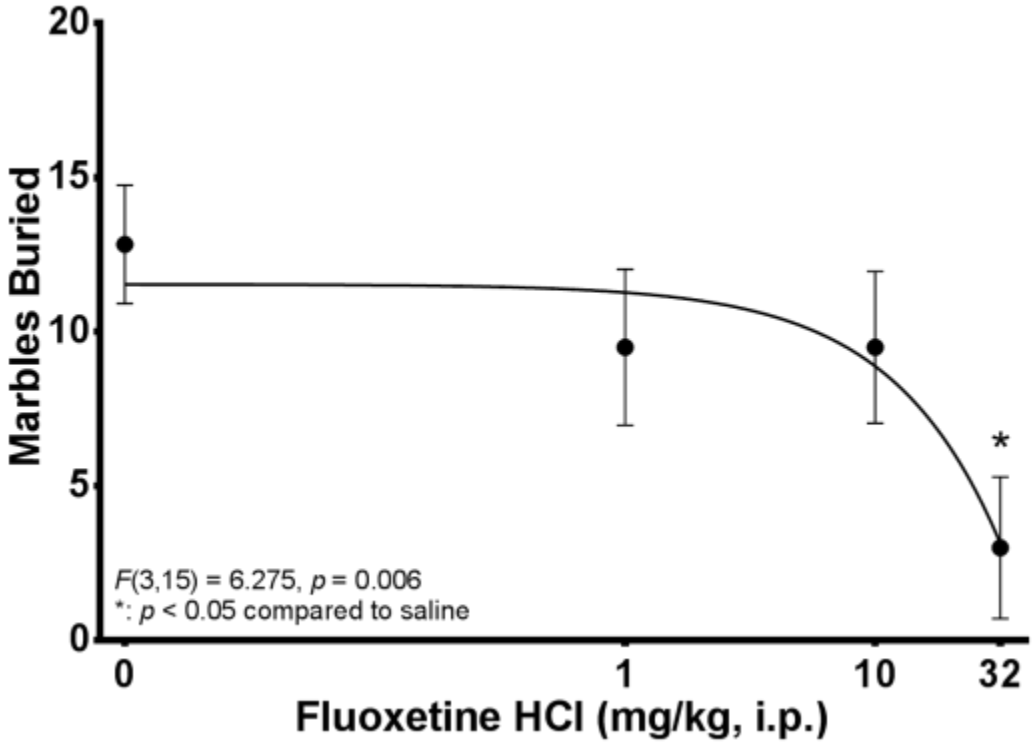

Figure 15: Pilot test of marble burying with fluoxetine HCl.

N=8 wildtype C57BL/6J mice. Significant effect of drug with decreased number of marbles buried at 32 mg/kg. Mean ± SEM.

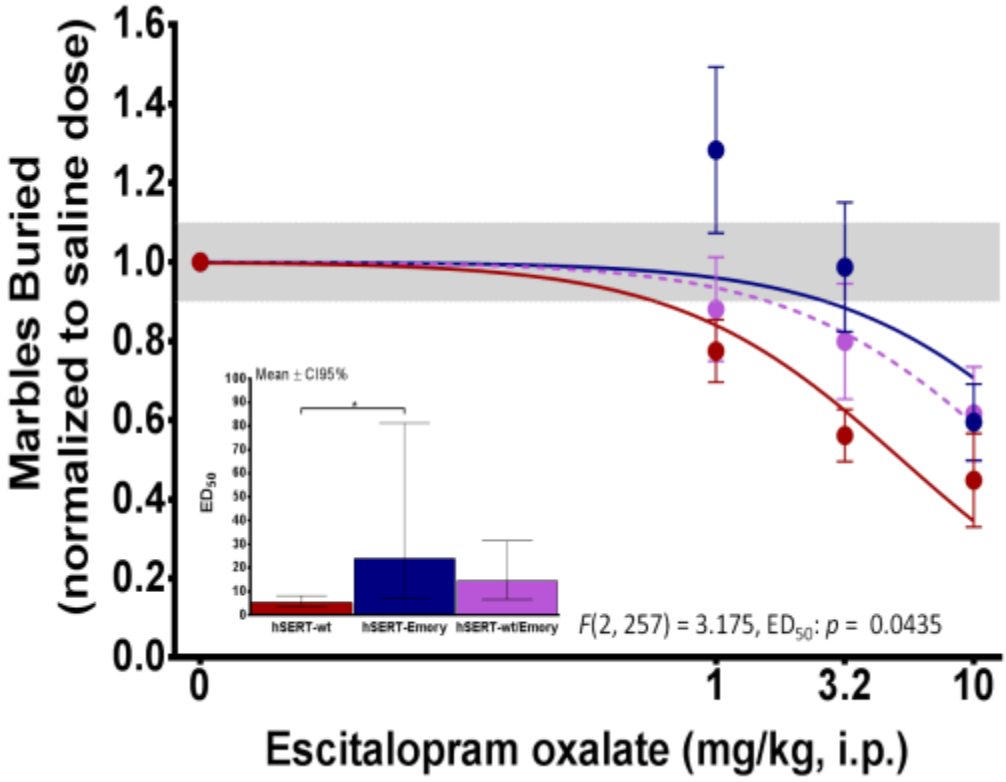

Figure 16: Marble burying with escitalopram oxalate in knockin mice.

Red: hSERT-wt, N=17; Blue: hSERT-Emory, N=26; Purple: hSERT-wt/Emory, N=22.

Gray bar indicative of variation in saline dose prior to normalization. *: $p < 0.05$. Mean ± SEM.

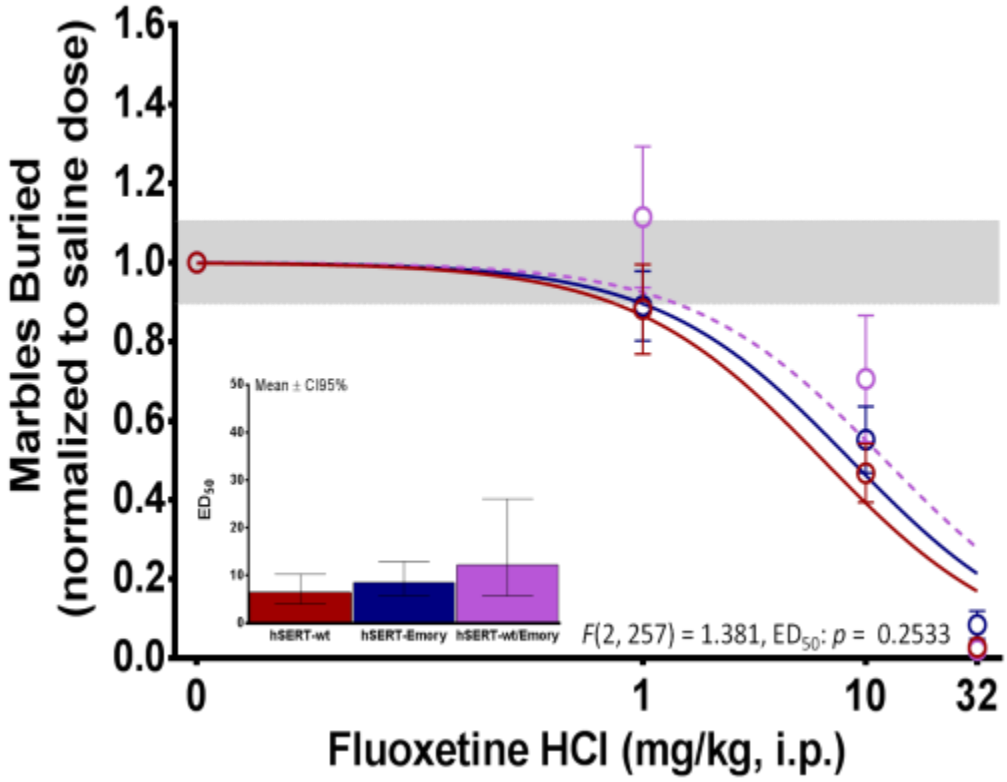

Figure 17: Marble burying with fluoxetine HCl in knockin mice.

Red: hSERT-wt, N=17; Blue: hSERT-Emory, N=26; Purple: hSERT-wt/Emory, N=22.

Gray bar indicative of variation in saline dose prior to normalization. Mean ± SEM.

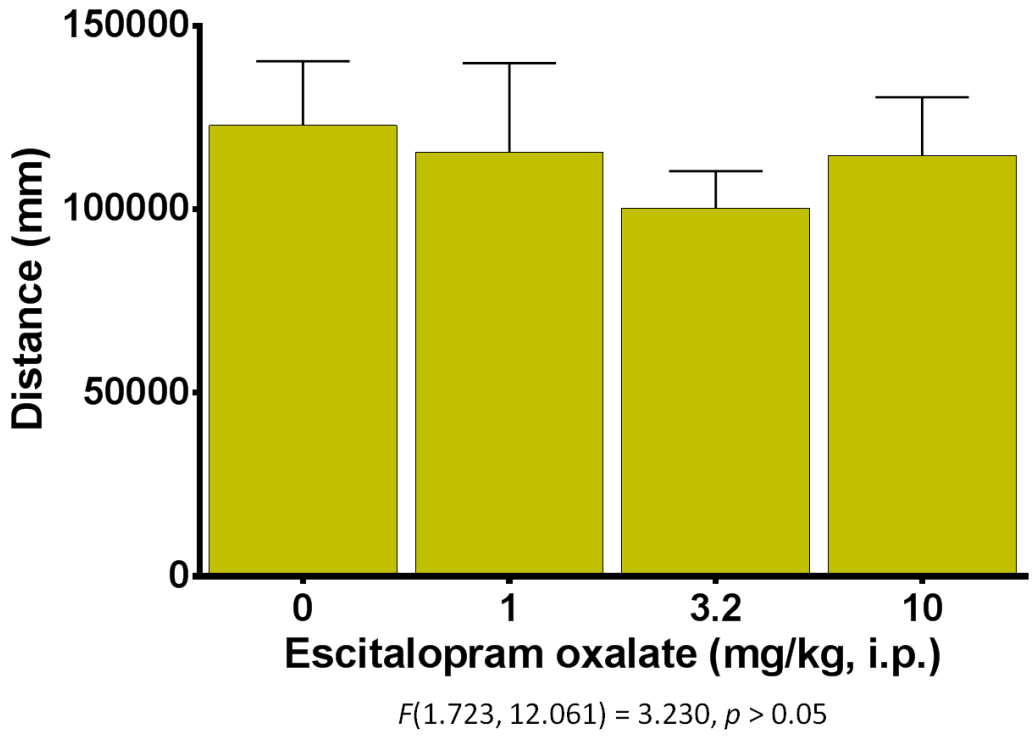

Figure 18: Locomotion during pilot escitalopram oxalate MB.

No significant effect of escitalopram on distance travelled. N=8 C57BL/6J mice. Mean ± SEM.

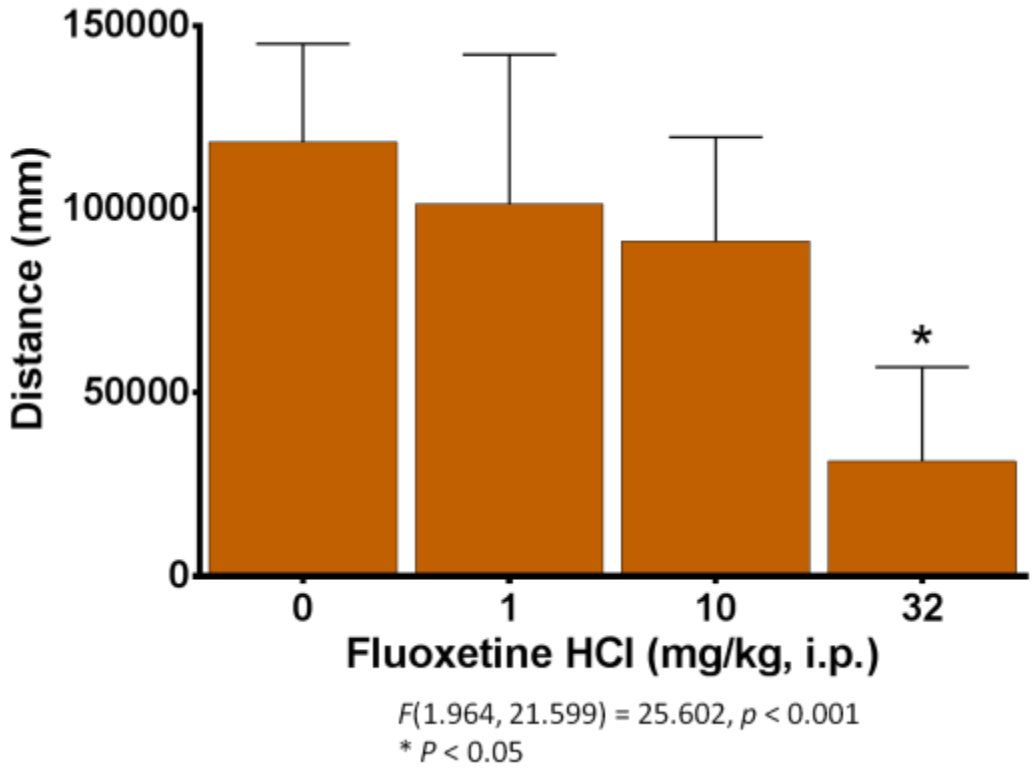

Figure 19: Locomotion during pilot fluoxetine HCl MB.

Significant effect of fluoxetine on distance travelled at 32 mg/kg. N=8 C57BL/6J mice.

Mean ± SEM.

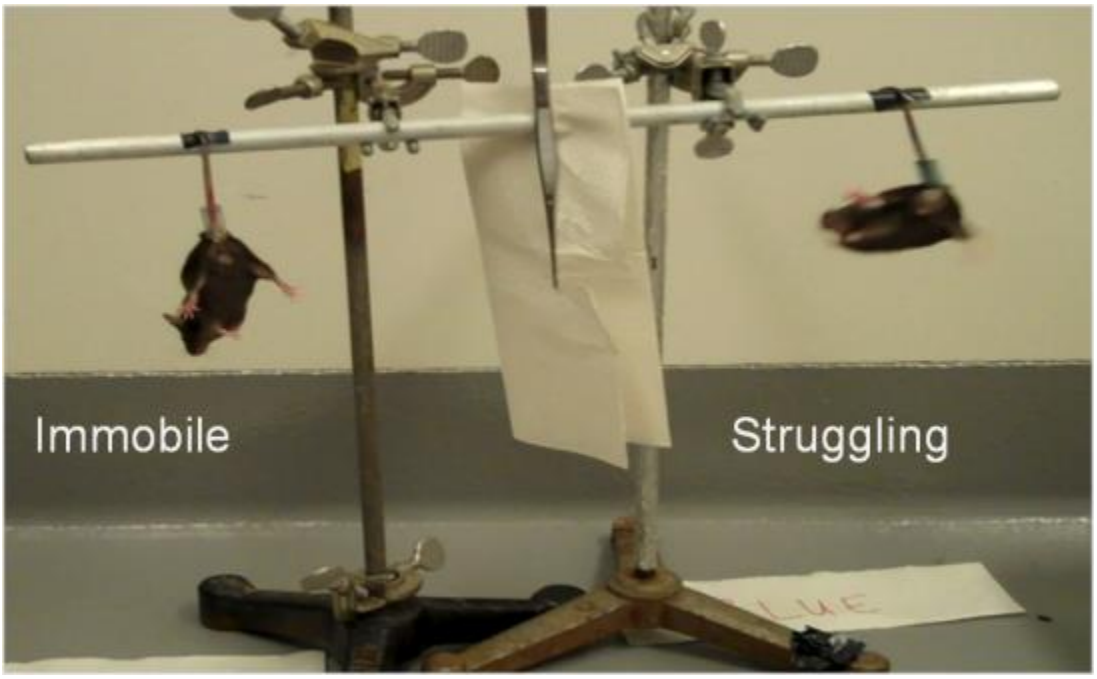

Figure 20: Tail suspension test view.

Left-side mouse is immobile and the right-side mouse is struggling. Note the plastic tubing on the tail to prevent tail climbing.

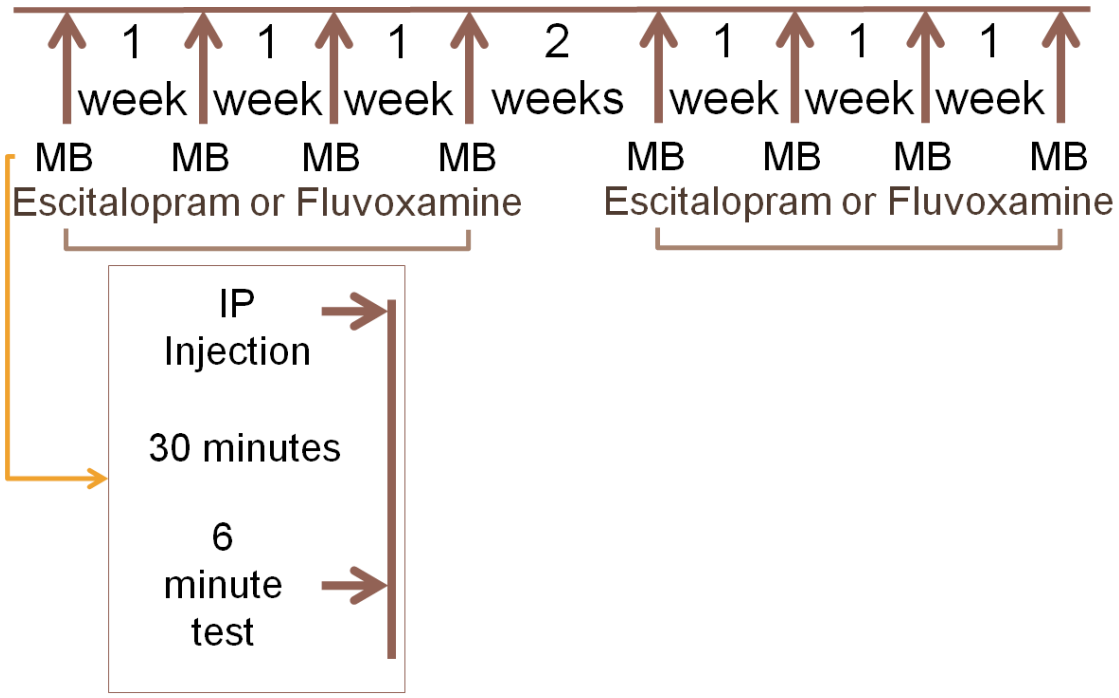

Figure 21: Tail suspension test schematic.

Each mouse had 8 TST sessions over 9 weeks. Each mouse had the 4 doses of escitalopram oxalate (0-10 mg/kg) or 4 doses of fluvoxamine maleate (0-32 mg/kg) over the first 4 weeks, followed by 2 week washout period, then 4 weeks using the other SSRI. Per individual session the drug was administered 30 minutes prior to the test via i.p. injection.

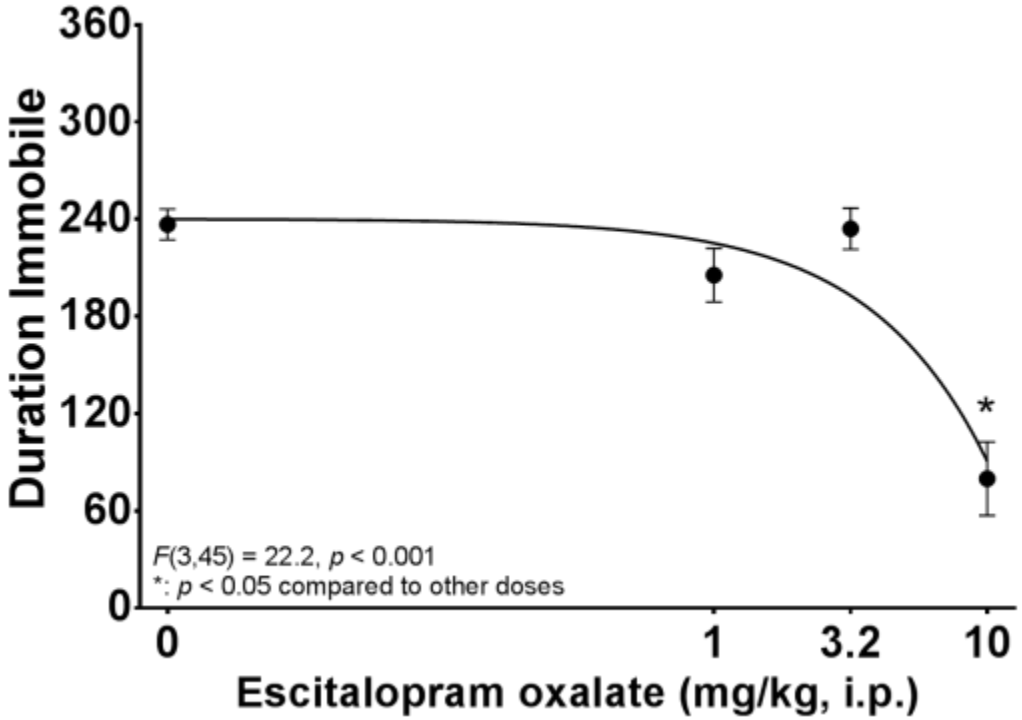

Figure 22: Tail suspension test escitalopram oxalate pilot in C57BL/6J mice.

There is a significant decrease in time immobile at 10 mg/kg escitalopram oxalate. Saline N=20; 1 mg/kg N=9; 3.2 and 10 mg/kg N=8. Mean ± SEM.

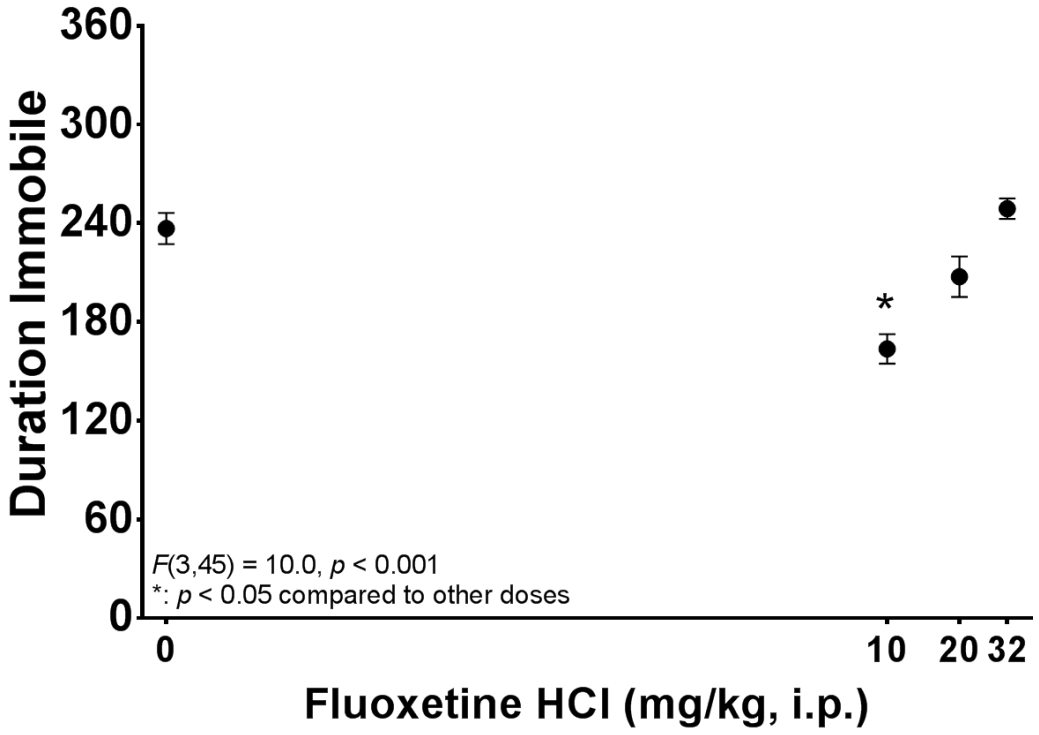

Figure 23: Tail suspension test fluoxetine HCl pilot in C57BL/6J mice.

There is a significant decrease in time immobile at 10 mg/kg fluoxetine HCl. Saline values pooled over three testing weeks, when the individual week of the 10 mg/kg fluoxetine HCl testing is compared to its matched week saline group, there is no significant effect. Saline N=20; 10 mg/kg N=7; 20 and 32 mg/kg N=8. Mean ± SEM.

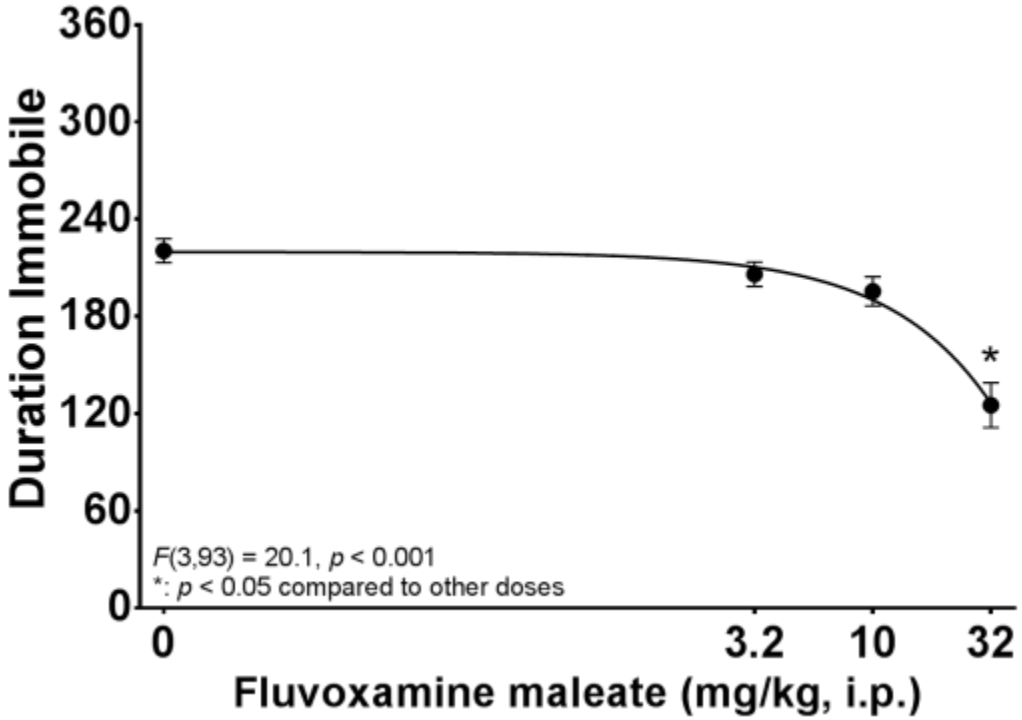

Figure 24: Tail suspension test fluvoxamine maleate pilot in C57BL/6J mice. There is a significant decrease in time immobile at 32 mg/kg fluvoxamine maleate. Saline, 3.2 mg/kg, 32 mg/kg N=24; 10 mg/kg N=21. Mean ± SEM.

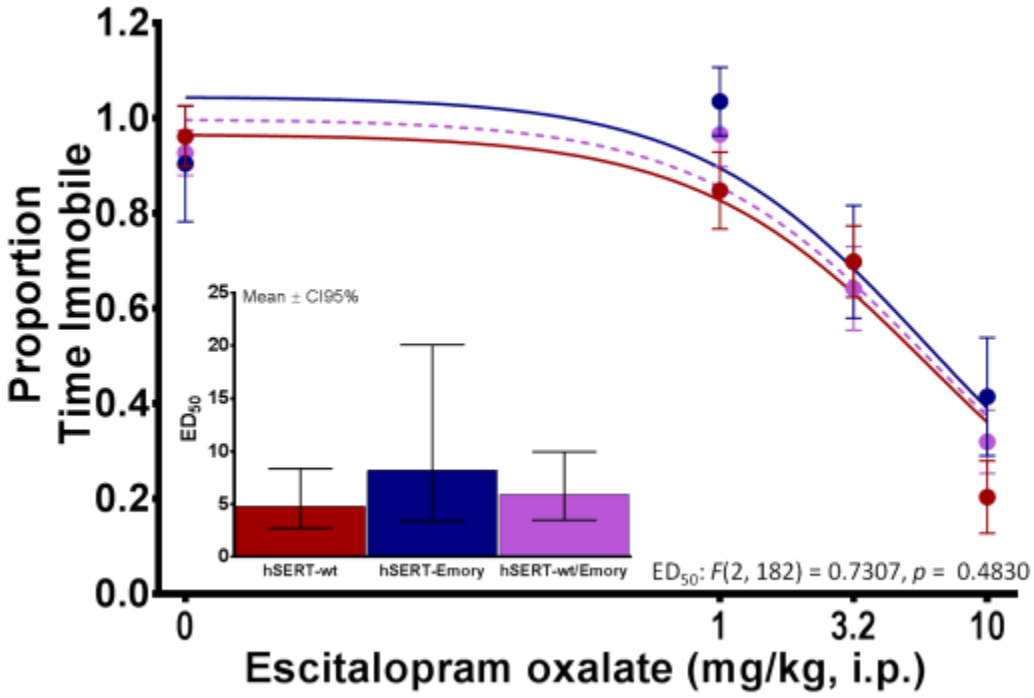

Figure 25: Tail suspension test with escitalopram oxalate in knockin mice, duration immobile.

Red: hSERT-wt, N=15; Blue: hSERT-Emory, N=11; Purple: hSERT-wt/Emory, N=21.

Mean ± SEM.

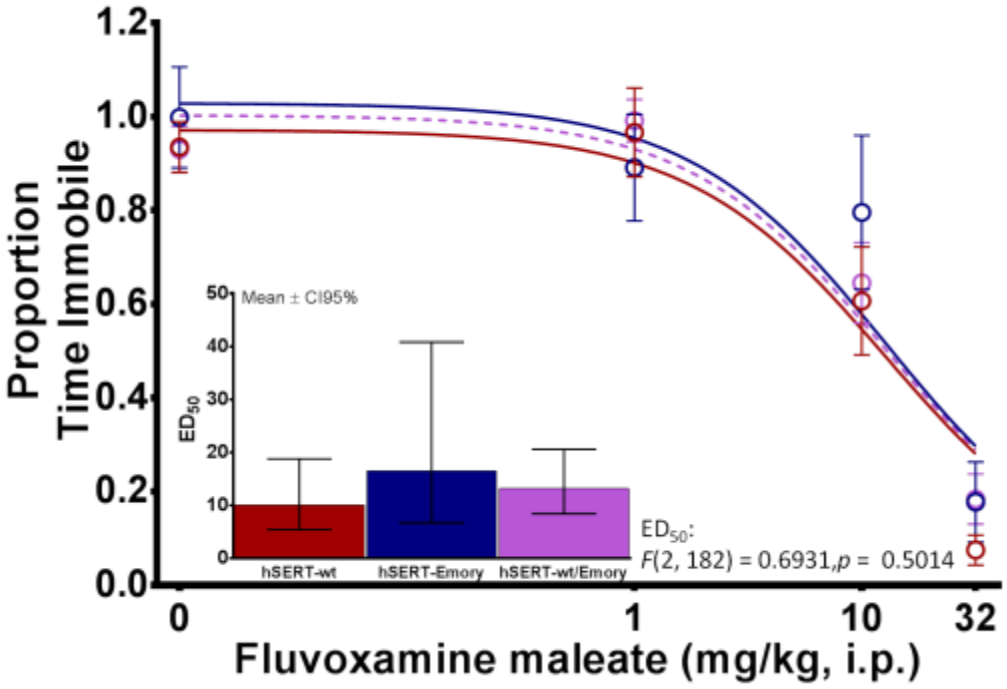

Figure 26: Tail suspension test with fluvoxamine maleate in knockin mice, duration immobile.

Red: hSERT-wt, N=15; Blue: hSERT-Emory, N=11; Purple: hSERT-wt/Emory, N=21. Mean ± SEM.

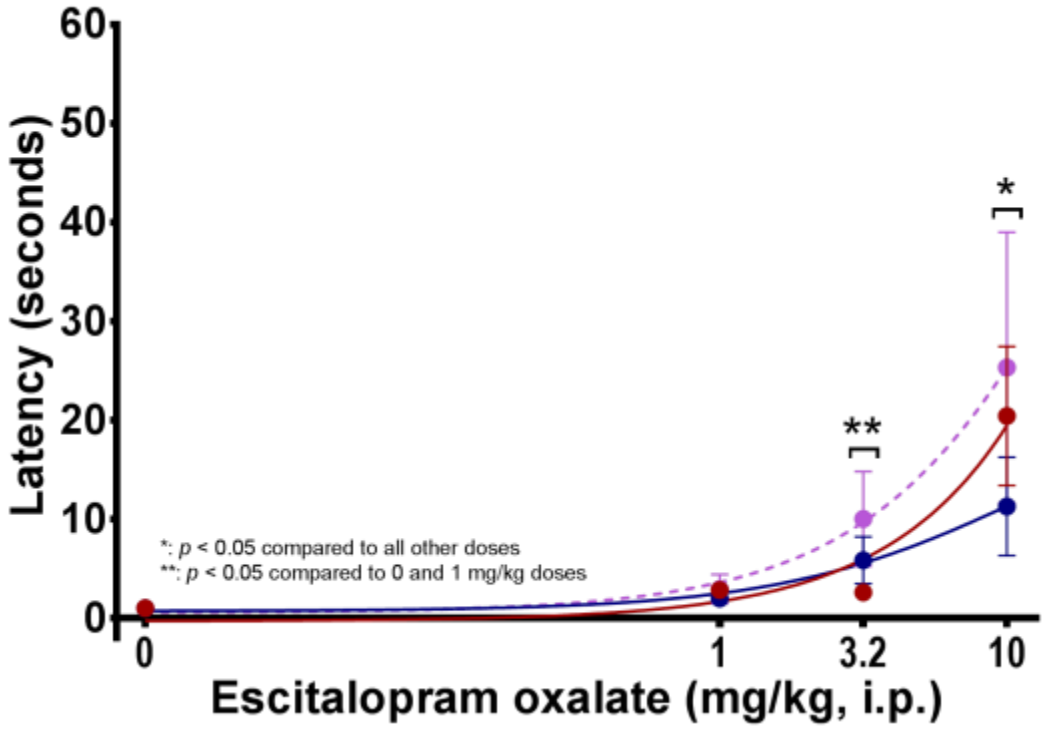

Figure 27: Tail suspension test with escitalopram oxalate in knockin mice, latency to first immobility event.

Red: hSERT-wt, N=15; Blue: hSERT-Emory, N=11; Purple: hSERT-wt/Emory, N=21.

Mean ± SEM.

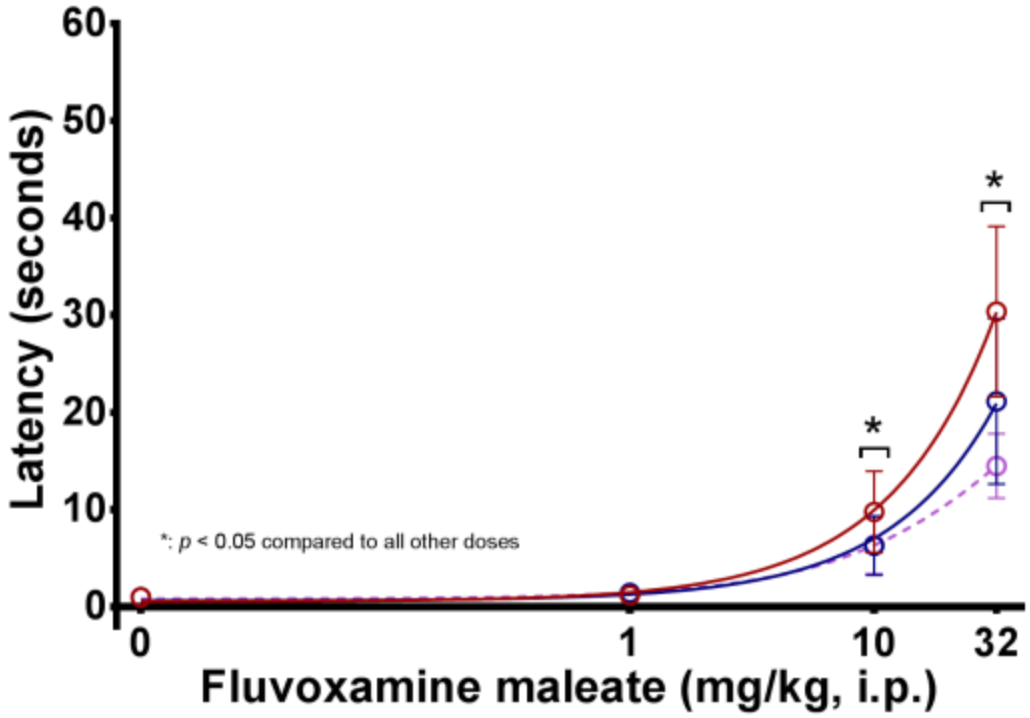

Figure 28: Tail suspension test with fluvoxamine maleate in knockin mice, latency to first immobility event.

Red: hSERT-wt, N=15; Blue: hSERT-Emory, N=11; Purple: hSERT-wt/Emory, N=21.

Mean ± SEM.

Chapter 6: Discussion and Conclusions

Abstract

The microdialysis, MB, and TST findings fit together to suggest that although there is a modest effect in MB, overall the presence or absence of the escitalopram allosteric does not present a robust role *in vivo*. Future study could elucidate potential regional specificity in the site's role, it is unlikely to provide translatable results in the care and treatment of patients with psychiatric disorders. Therefore, the results of the microdialysis and TST studies outweigh the modest result in the MB studies and the hypothesis that the allosteric site has an *in vivo* role in escitalopram's mechanism of action is not supported.

General Discussion

Escitalopram is a widely prescribed SSRI to treat psychiatric disorders. Identification of a secondary binding site on the hSERT spurred interest in what this site is responsible for in escitalopram's activity at the hSERT. Understanding the putative allosteric action of escitalopram could potentially open a new pathway to target pharmaceutically. *In vitro* work suggested removing the allosteric site altered the kinetics of escitalopram and hSERT interaction. *In vivo* work in rodents and clinical work in humans suggested that *R*-citalopram blunts the effects of escitalopram. Paired together these findings lead to the hypothesis that the allosteric site is the locus of escitalopram activity that alters how primary site bound escitalopram inhibits the hSERT. To study this directly, the hSERT-wt and hSERT-Emory mice provided the tools to compare the presence and absence of the allosteric site *in vivo*.

Autoradiography was used to confirm the expression of the hSERT knockin protein and thus how to use the knockin mice. Microdialysis studies examined the physiological role of the allosteric site in the ventral hippocampus. MB and the TST were used to study the behavioral output of the allosteric site. Ideally these studies would have converged to an answer of what is the role of the escitalopram allosteric site on the hSERT. Yet these studies to do not come to a robust conclusion, rather they highlight that, at most, the allosteric site has a fine tuning role in the pharmacology of escitalopram.

In characterizing the knockin mouse lines, autoradiography revealed that hSERT expression was reduced compared to that of mSERT in typical C57BL/6J mice. The reduced expression could have played into the microdialysis and behavioral results;

however it does not seem that it was impactful. Further, a lack of difference in expression between the knockin lines maintains the ability of the two knockin lines to be compared to each other. While there may be some differences between the knockin lines and wildtype mice, it is ultimately the role of the knockin lines to be compared to each other to screen the role of the allosteric site. And the autoradiography data indicated that the mice are reasonable tools.

Taken together, the microdialysis and behavioral data create an interesting picture of how the allosteric site on the hSERT plays a role in escitalopram's mechanism of action. The hypothesis was that removing the allosteric site on the hSERT would result in a blunting of escitalopram's effects so that a larger dose would be required to achieve the same level of effect as in hSERT-wt mice. The MB data supported this hypothesis, with the modest rightward shift in ED_{50} values for escitalopram in the absence of the allosteric site. The microdialysis and TST data, however, did not support the hypothesis, and rather suggested no role of the allosteric site in escitalopram's effects in those studies. How these data fit together to further characterize escitalopram's allosteric site on the hSERT will be discussed in more detail below.

The escitalopram allosteric site is hypothesized to cause an increase in extracellular serotonin. This would be in addition to the buildup of extracellular serotonin that results from inhibiting the transporter via the primary binding site. Because the transporter is successfully inhibited via primary binding, the additional amount of serotonin should not be of a very large magnitude. Consequently this small amount of extra serotonin is likely only capable of making a fine level of effect or is only effective in junctures very sensitive to serotonin levels. In light of this, as previously discussed,

MB could be sensitive to fine tuning of serotonin, whereas TST may not be, allowing for the measured pattern of escitalopram effects. Had the microdialysis experiments revealed in the ventral hippocampus there is a difference in extracellular serotonin levels between the hSERT-wt and hSERT-Emory mice after escitalopram challenge this may have explained the MB result. However, there was no difference in microdialysis. Instead this suggests that the MB effect is not entirely hippocampal-linked.

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

What is interesting about the MB data is it does seem to be in line with clinical work which identified escitalopram to have a significant but modest superiority to citalopram (Ali & Lam, 2011; Gorman et al., 2002; Lepola et al., 2004; Montgomery et al., 2007; Montgomery, Loft, Sánchez, Reines, & Papp, 2001; Moore, Verdoux, &

Fantino, 2005; Waugh & Goa, 2003). In clinical studies and meta-analyses of these studies the data shows that patients respond more quickly to escitalopram than to citalopram, and faster than placebo and other antidepressants. Yet the separation between escitalopram and other compounds does not have a large magnitude and patients still respond to the other compounds. The MB effect due to the presence or absence of the allosteric site is measured as an acute response in mice and is clearly not an exact model of any particular psychiatric disorder, rather a screen of compounds that may relieve psychiatric symptoms. Perhaps MB in mice with acute drug challenge is sensitive to correlates of faster response to SSRIs in humans. And perhaps the TST after acute drug challenge can only screen if a compound is able to evoke a response in humans. While this is purely speculative, it would be an explanation for why there was a different conclusion for the two behavioral tests. It is also a reminder that behavioral tests in mice are only able to screen and measure aspects of complex human disorders, and that an individual finding is not stand alone evidence that can be directly translated to humans.

The MB, TST and microdialysis data led to the overall conclusion that the allosteric site has some limited effects on escitalopram action *in vivo*. Yet those effects are not far-reaching and overall comparison of the hSERT-wt and hSERT-Emory mice indicates that *in vivo* the allosteric site is not a key player in the mechanism of action of escitalopram.

Conclusions

Together the physiological and behavioral data suggest that there is a modest effect of the allosteric site in escitalopram action, but it is not a robust effect that supports further pursuing of the site as a target of pharmacological merit *in vivo*. While the MB

finding on its own is interesting, the lack of corroboration from another behavioral screen of SSRI activity and microdialysis suggests that the MB finding does not measure an important effect.

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

Future Directions

While the investigation of the escitalopram allosteric site on the hSERT using a knockin mouse suggests that ultimately the allosteric site does not have a robust *in vivo* role, the knockin mice could still be of value. The mice could be used to further examine the physiological and behavioral questions described previously, or to examine other questions about the allosteric site.

To further examine the role of the escitalopram allosteric site using the methods herein, *R*-citalopram could be included in the studies. While escitalopram alone does not appear to have a robust effect on the allosteric site, perhaps *R*-citalopram does. Escitalopram's ability to inhibit the transporter is disrupted in the presence of *R*-citalopram, a result that has previously been discussed both *in vitro* and *in vivo*. Perhaps this effect lies more in the *R*-enantiomer rather than the *S*-enantiomer, despite escitalopram's greater affinity for the hSERT. To this end, the interaction of the enantiomers at an allosteric-null hSERT, although a different set of mutations to remove the allosteric site, has been examined (Jacobsen et al., 2014). This study sought to identify clinically relevant doses of escitalopram and *R*-citalopram, and then administered the compounds via intravenous injection while performing frontal cortex microdialysis. They found that in the allosteric-null hSERT mice the addition of *R*-citalopram augmented extracellular serotonin levels compared to escitalopram alone. The hSERT-Emory mice could be used to confirm this finding.

Jacobsen et al. (2014) also examined their mice using the MB and TST. Using low doses of escitalopram, chosen to mimic clinical brain concentrations, they found *R*-citalopram augmented escitalopram's effects in the TST only in hSERT-wt mice, and no effect in the MB or on the allosteric-null mice. This could suggest that the larger doses used to study the hSERT-Emory mice were possibly dosed beyond the clinical range, and thus any effects seen in the MB and TST may be an artificial result due to overloading the serotonergic system. A future direction could be to see if the MB and TST results in the hSERT-Emory mice hold true at the clinically relevant doses suggested.

Several attempts have been made to make a compound that specifically targets the allosteric site. SoRI-6238 was identified to be an allosteric modulator of the SERT (Nandi, Dersch, Kulshrestha, Ananthan, & Rothman, 2004). While published work does not indicate that this compound has been pursued, screening it with the hSERT-wt and hSERT-Emory mice would identify if it was acting at the escitalopram allosteric site, and further as having an *in vivo* role via the site. Citalopram analogues made to target the allosteric site on the SERT have been studied *in vitro*, but the knockin lines could further define the value of the compounds *in vivo* (Banala et al., 2013).

In these studies the hSERT-wt mice served as a control for the hSERT-Emory mice. These mice had an intact hSERT protein, but they accounted for the effects of manipulating the mouse genome. Yet the hSERT-wt mice can serve as more than a control for the hSERT-Emory mice, rather they could be a stand alone tool to study the human transporter in a malleable model organism. Further, the decreased expression of the knockin protein compared to the mouse transporter could serve as a model of decreased SERT expression. Changes in SERT expression are known after SSRI administration, perhaps the hSERT mice could be useful in examining the effects of decreased SERT expression. Additionally, further characterization of the hSERT-wt mice response in typical SSRI screens could identify how these mice may be different from wildtype mice and how the MB results discussed herein could be further interpreted.

Finally, earlier discussion suggested that the role of the allosteric site may have regional specificity in action. That specificity may be highlighting that the MB and TST utilize different pathways in the mouse's brain. In humans SSRIs are known to take time before their therapeutic effects, whereas in mice there is an acute effect and the

physiological effect of inhibiting the SERT is not on the order of weeks as the therapeutic effect. Serotonin autoreceptors have been implicated in MB, as that agonist of these receptors causes an effect similar to that of SSRIs, but this effect is reduced when the autoreceptors are antagonized (Matsushita et al., 2005). Further, antagonism of the serotonin autoreceptors enhances the effects of citalopram by increasing extracellular serotonin levels in the frontal cortex of rats, but only non-selective antagonists had this effect in the dorsal hippocampus, indicative of a regional specificity (Gundlah, Hjorth, & Auerbach, 1997). Adding a layer of serotonin autoreceptor antagonism as well as more locations to the microdialysis studies could elucidate a regional specificity for the role of escitalopram's allosteric site, and potentially map the differences between the MB and TST results.

Electrophysiology could augment microdialysis studies. Recording in the dorsal raphe, previous work in rats identified that *R*-citalopram dampens the inhibition effect of escitalopram on neuron firing (Mnie-Filali et al., 2006). Such recordings are also possible in mice and have previously been used in investigations of the SERT, specifically SERT knockout mice, in both the hippocampus and dorsal raphe (Gobbi, Murphy, Lesch, & Blier, 2001). While microdialysis studies measure the direct effect of escitalopram on serotonin uptake, electrophysiological studies would be more indirect and incorporate measures of how the serotonin cell firing changes as serotonin uptake is inhibited, and this technique can be more sensitive to small changes. The hSERT-wt and hSERT-Emory mice are a nice tool that could further the prior electrophysiological studies in rats. And such studies could then be paired with microdialysis studies to map potential regional effects of the allosteric site.

Final Remarks

These studies of the characterization of the hSERT-wt and hSERT-Emory mice and the subsequent comparison of the knockin lines to examine the *in vivo* role of the escitalopram allosteric site on the hSERT came to the conclusion that the allosteric site does not present a robust *in vivo* role. Consequently it should not be an active target of interest for future pharmaceutical study. The knockin lines themselves remain a valuable asset to examine any future questions about the allosteric site, and the hSERT-wt mice alone can be of use to examine and manipulate the hSERT protein in an experimental system. As of now, though, the allosteric site for escitalopram has run its course as a novel locus of interest.

Appendix A: Knockin Gene Insertion

The cDNA sequence for the hSERT-wt knocked into the mouse genome is:

atggagacg acgcccttga attctcagaa gcagctatca gcgtgtgaag atggagaag
ttgtcaggaa aacggagttc tacagaaggt tgttcccacc ccaggggaca aagtggagtc
cgggcaaata tccaatgggt actcagcagt tccaagtcct ggtgcgggag atgacacacg
gcactctatc ccagcgacca ccaccaccct agtggctgag cttcatcaag gggaacggga
gacctggggc aagaaggtgg atttccttct ctcagtgatt ggctatgctg tggacctggg
caatgtctgg cgcttcccct acatatgtta ccagaatgga gggggggcat tcctcctccc
ctacaccatc atggccattt ttgggggaat cccgctcttt tacatggagc tcgcactggg
acagtaccac cgaaatggat gcatttcaat atggaggaaa atctgcccga ttttcaaagg
gattggttat gccatctgca tcattgcctt ttacattgct tcctactaca acaccatcat
ggcctgggcg ctatactacc tcatctcctc cttcacggac cagctgccct ggaccagctg
caagaactcc tggaacactg gcaactgcac caattacttc tccgaggaca acatcacctg
gaccctccat tccacgtccc ctgctgaaga attttacacg cgccacgtcc tgcagatcca
ccggtctaag gggctccagg acctgggggg catcagctgg cagctggccc tctgcatcat
gctgatcttc actgttatct acttcagcat ctggaaaggc gtcaagacct ctggcaaggt
ggtgtgggtg acagccacct tcccttatat catcctttct gtcctgctgg tgaggggtgc
caccctccct ggagcctgga ggggtgttct cttctacttg aaacccaatt ggcagaaact
cctggagaca ggggtgtgga tagatgcagc cgctcagatc ttcttctctc ttggtccggg
ctttggggtc ctgctggctt ttgctagcta caacaagttc aacaacaact gctaccaaga
tgccctggtg accagcgtgg tgaactgcat gacgagcttc gtttcgggat ttgtcatctt
cacagtgctc ggttacatgg ctgagatgag gaatgaagat gtgtctgagg tggccaaaga
cgcaggtccc agcctcctct tcatcacgta tgcagaagcg atagccaaca tgccagcgtc
cactttcttt gccatcatct tctttctgat gttaatcacg ctgggcttgg acagcacgtt
tgcaggcttg gaggggggtga tcacggctgt gctggatgag ttcccacacg tctgggccaa
gcgccgggag cggttcgtgc tcgccgtggt catcacctgc ttctttggat ccctggtcac
cctgactttt ggaggggcct acgtggtgaa gctgctggag gagtatgcca cggggcccgc
agtgctcact gtcgcgctga tcgaagcagt cgctgtgtct tggttctatg gcatcactca
gttctgcagg gacgtgaagg aaatgctcgg cttcagcccg gggtggttct ggaggatctg
ctgggtggcc atcagccctc tgtttctcct gttc**atcatt** tgcagttttc tg**atgagc**cc
gccacaacta cgacttttcc aatataatta tccttactgg **agtatc**atct tgggttactg
cataggaacc tcatctttca tttgcatccc cacatatata gcttatcggt tgatcatcac
tccagggaca tttaaagagc gtattattaa aagtattacc ccagaaacac caacagaaat
tccttgtggg gacatccgct tgaatgctgt gtaa

The bold underlined bases are the locations of the point mutations changed to create the hSERT-Emory sequence, correlating with the paired amino acid changes:

II/552,553/VT	atcatt → gtcact
MS/558,559/SN	atgagc → tcgaac
SI/574,575/TT	agtatc → actacc

The final 72 bases overlap with the mSERT codon 14. This facilitated the homologous recombination of the hSERT constructs into the mouse genome (Figure 29).

The hSERT constructs were inserted to the mouse genome by first placing these constructs into the pcDNA3 mammalian expression vector between the Xho1 and Xb1 sites. To facilitate this insertion, the hSERT construct was PCR amplified with the following upstream sequence from the start codon:

*CACTGGCGGCCG*CTCGAGGGATCCATAGAAGGCAGG**ATG**

The italicized bases are overlap from the vector sequence and the bolded bases are the start codon overlapping the hSERT sequence (as previous). Underlined is noncoding sequence and the plain text is the Xho1 site.

Similarly, after the taa stop codon in the hSERT sequence the Xba site was included with a 3' PCR primer:

TAAGGTACCAAGCTCTAGA*GGGCCCTAT*

Again, the italicized bases are from the vector, bolded bases are from the hSERT sequence, underlined is noncoding, and plain text is the Xba site.

Overlapping 5' "A" box and 3' "B" box were also included in the construct sequence to facilitate homologous recombination into the mouse genome at the mSERT locus. The "A" box and "B" box sequences added to the hSERT cDNA sequence in the construct inserted into the pcDNA3 vector.

The hSERT-wt or hSERT-Emory construct was inserted into the pcDNA3 vector and then inserted into mouse embryonic stem cells (ES). A neomycin resistance cassette was also part of the vector, and neomycin resistance was used to select ES for use. After successfully inserting the construct, the cassette was removed. The selected ES were then

inserted into pseudopregnant female 129S6/SvEvTac mice. Chimeric pups were then bred to a stable line of hSERT/mSERT heterozygous mice, for each hSERT-wt and hSERT-Emory (Figure 30). These mice were then used to establish homozygous lines of hSERT-wt and hSERT-Emory, as described in Chapter 2.

Figures

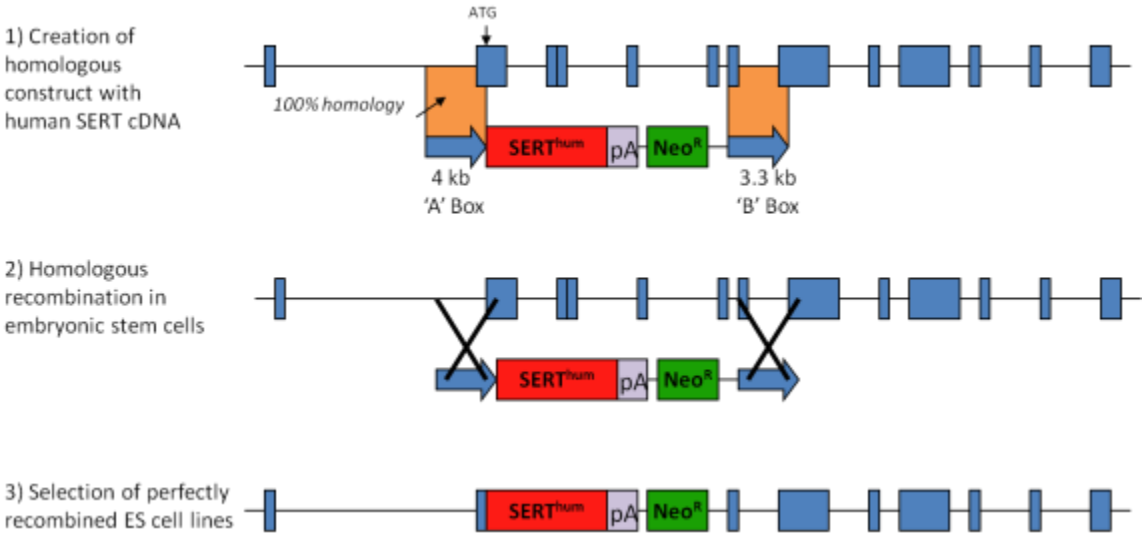

Figure 29: Cartoon of homologous recombination of the hSERT construct into the mouse genome.

Note that before and after the SERT sequence are regions homologous to the mouse genome. The Neo cassette was removed after insertion.

Figure 30: Chimeric pups born summer 2007.

These pups have the hSERT-Emory construct. First hSERT-wt chimeras born spring 2008.

Appendix B: Genotyping Protocol

Tail snips were obtained from each mouse in the knockin colony. DNA was extracted via a phenol-chloroform extraction method. After DNA was extracted and dried it was reconstituted in molecular grade water and stored at 4 °C.

Primers that could discriminate between mSERT, hSERT-wt, and hSERT-Emory are essential for identification of mice during production of the mice, breeding, and experimentation. Both hSERT allele primers were developed from the hSERT constructs inserted into the mouse genome. The hSERT-wt and hSERT-Emory 5’ primers overlapped in location, but included 4 of point mutations that differentiated the hSERT genes. These allele specific primers were able to discriminate between the two similar alleles to properly identify the genotype of the mouse.

The hSERT primers:

3' hSERT: 5' ttacacagcattcaagcggatgtcc 3' (25 bp)

5' hSERT-wt: 5' ctgttcatcatttgcagttttctgat 3' (26 bp)

5' hSERT-Emory: 5' ctgttcgtcacttgcagttttctgtc 3' (26 bp)

The mSERT primer was used only while establishing the mouse lines; after the mSERT gene was removed from the mouse lines and all breeder mice were confirmed to have only the humanized SERT genes it was no longer necessary to confirm the absence of the mSERT gene. The choice of the mSERT primer sequences was provided by Marc Caron’s group (Duke University). The mSERT primers were a trio that would amplify both hSERT and mSERT, but the primers could not differentiate between the hSERT alleles and the hSERT and mSERT products were of different size making them easy to distinguish from one another. The hSERT product was not visualized under the PCR

protocol utilized, so effectively only the mSERT product could be identified using this primer set.

The mSERT primers are:

Fs1: 5’- tcgctgtgtcttggttctatggca-3’ (24 bp)

P19: 5’-gtacaagcgctggggatgaagcgcc-3’ (24 bp)

P17: 5’- gctcacgtcagctacccaggac-3’ (21 bp)

Successful PCR amplification was achieved using GoTaq Green Master Mix (Promega). The following master mix recipe was used:

10 uL/sample GoTaq Green

1 µL/sample each primer at 25 µM (Fs1, P19, P17 for mSERT; 3’ hSERT and the appropriate 5’ hSERT for either hSERT-wt or hSERT-Emory)

Water to total volume 24 µL/sample

For each sample, 1 µL of sample DNA was added to 24 µL of the master mix solution in PCR tubes. The sample in the master mix solution was kept on ice until a hot start of the thermocycler, set to the following conditions:

1. 95 °C 4 minutes
2. 95 °C 30 seconds
3. 62 °C 30 seconds
4. 72 °C 30 seconds
5. Repeat steps 2-4 35 times
6. 72 °C 5 minutes
7. 4 °C hold

After the end of the PCR run, samples are placed in individual lanes of a 1.5% agarose gel. DNA bands are then imaged under UV light. Each hSERT primer set produces about a 250 bp product, while the mSERT product is about 600 bp.

Each mouse was genotyped in this manner. An example may be seen in Figure 31. Positive controls for the hSERT-wt and hSERT-Emory primers were maxiprep constructs. The mSERT control was from a mouse known to have no knockin SERT. The negative control was master mix without any added DNA.

Figure

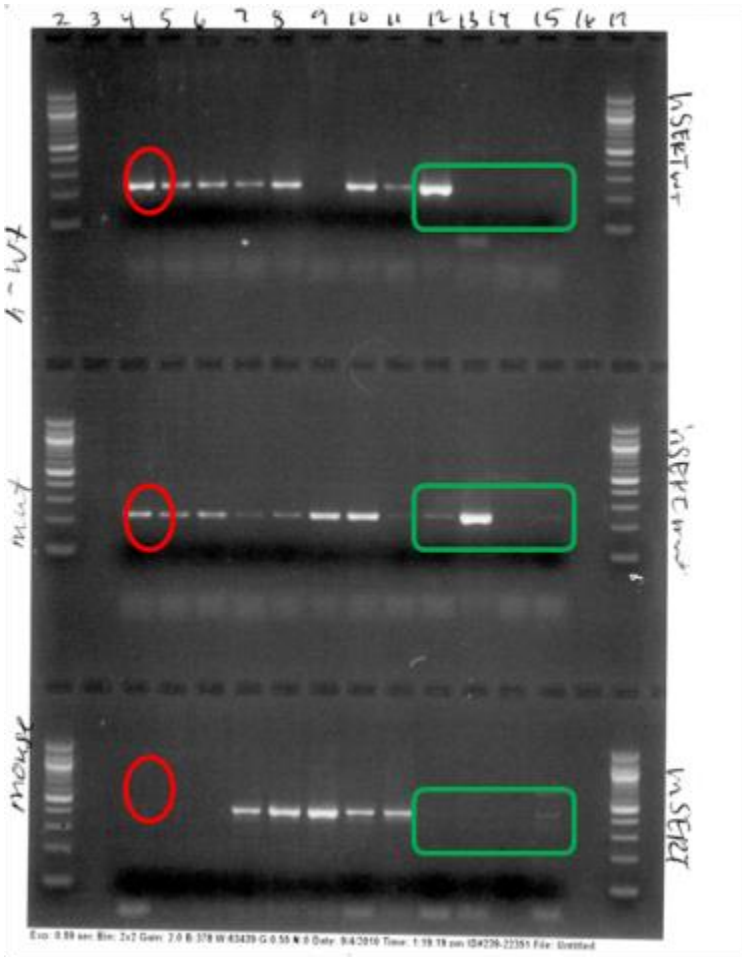

Figure 31: Representative agarose gel of PCR products.

Row 1 is with hSERT-wt primers. Row 2 is with hSERT-Emory primers. Row 3 is mSERT primers. Circled in green, lane 12 has the hSERT-wt positive control, lane 13 has the hSERT-Emory positive control, lane 14 has the mSERT positive control, and lane 15 is the negative control (blank). Each lane, 4-11, is a single mouse run for each primer set. Note lane 4, circled in red, which is heterozygous for the hSERT genes.

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