EXPLORING SEROTONIN 1B AUTORECEPTOR MEDIATED REGULATION OF SEROTONIN TRANSPORTER IN FEMALE MICE

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Pharmacology and Toxicology at Virginia Commonwealth University

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List of Abbreviations

5-HT = Serotonin

SERT = Serotonin Transporter

5-HT_{1B}/$5HT_{1B}$ R = Serotonin 1B autoreceptor

GSKα/β = Glycogen Synthase Kinase 3α/β

MDD = Major Depressive Disorder

OCD = Obsessive Compulsive Disorder

ADHD = Attention Deficit Hyperactivity Disorder

ASD = Autism Spectrum Disorder

DAT = Dopamine Transporter

NET= Norepinephrine Transporter

NE = Norepinephrine

DA = Dopamine

TCA = TriCylic Antidepressants

SSRI = Selective Serotonin Reuptake Inhibitors

SNRI = Selective Norepinephrine Reuptake Inhibitors

MAO I = Monoamine Oxidase Inhibitors

GPCR = G-protein Coupled Receptors

SLC = Solute Carrier Family

CAMKII = Calcium-calmodulin dependent kinase II

Akt = Protein Kinase B

PKC = Protein Kinase C

MAPK = Mitogen Activated Protein Kinase

ABSTRACT

EXPLORING SEROTONIN 1B AUTORECEPTOR MEDIATED REGULATION OF SEROTONIN TRANSPORTER IN FEMALE MICE

By

Sanyukta Jalihalkar, B.Pharm

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

at Virginia Commonwealth University

Director: Dr. Sammanda Ramamoorthy, Professor, Department of Pharmacology & Toxicology, School of Medicine

Rationale: Serotonin (5-hydroxytryptamine, 5-HT) is a crucial neurotransmitter involved in regulating mood, appetite, sleep, gastrointestinal motility, vasoconstriction and other physiological processes. Dysregulation of this serotonergic system has been implicated in several neuropsychiatric disorders, such as depression, anxiety, obsessive-compulsive disorder (OCD), and eating disorders. Though more than 15 different types of cell-surface 5-HT receptors exist to communicate the specific actions of 5-HT on target cells, a single gene encoding the Na+ and Cl--dependent 5-HT transporter (SERT) is responsible for extracellular 5-HT clearance in the brain and periphery. 5-HT_{1B} autoreceptors (5-HT_{1B} R) are strategically expressed on 5-HT terminals along with SERT and regulate 5-HT release as a feedback mechanism. Interestingly, 5-HT_{1B} R interacts with GSK3ß and at the same time it also inhibits 5-HT release. Moreover, activation or

inhibition of 5-HT_{1B} R regulates SERT function. Interestingly, our laboratory has demonstrated GSK3ß's role in modulating SERT via specific S44/S48 phosphorylation sites. While these findings collectively indicate a potential interplay among 5-HT_{1B} R and GSK3ß SERT phosphorylation to regulate extracellular 5-HT, the in vivo primary causal molecular link is unknown.

Objectives: To explore if 5-HT_{1B} autoreceptor activation by the 5-HT_{1B} R agonist CP-94253 influences SERT-mediated 5-HT clearance through GSK3ß- dependent SERT-Ser44/48 phosphorylation and investigate differences between brain regions and sexes.

Methods: Mice were injected with the 5-HT_{1B} R agonist CP-94253 (1mg/kg,i.p.). Brains were then dissected, and 5-HT uptake assays were performed to determine the effect of CP-94253 on SERT function. Kinetic analyses were conducted to assess transport velocity and substrate affinity. Assays were performed with region-specific crude synaptosomes obtained from female and male wild-type mice and compared with newly generated SERT-A44/A48 SERT Knock In mice lacking S44/48 phosphorylation sites. (S44/S48 substituted with non-phosphorylatable Ala in mSERT).

Results: Wild-type female mice injected with CP-94253 exhibited nonsignificant enhanced SERT-mediated 5-HT uptake in the striatum and hippocampus regions in females. Furthermore, preliminary investigations revealed that CP-94253 augmented substrate velocity in the hippocampus significantly, indicating increased transport capacity within wild-type female mice. Notably, mice with the SERT S44A/S48A Knock In mutation exhibit elevated SERT-mediated uptake even without any treatment. When these mice are exposed to CP94253, there is no further increase in 5-HT uptake. This suggests that the S44A/S48A sites on SERT play a crucial role in the GSK3ß-mediated phosphorylation pathway that regulates SERT.

Conclusions: The activation of 5-HT_{1B} autoreceptor leads to a somewhat enhanced 5-HT uptake via SERT in wild-type mice in the striatum and hippocampus regions in females, but it is not significant enough as compared to male mice. This difference between the control and treatment group in the SERT-mediated 5-HT uptake is lost in the SERT A44/A48 Knock In mice, where the phosphorylation site serine 44-48 is replaced with non-phosphorylatable alanine residues. Kinetic analyses further corroborate these findings. In female wild-type mice, CP94253 treatment results in a modest, though not statistically significant, increase in the maximal velocity (V_{max}) of 5-HT uptake in the striatum. More notably, a significant enhancement in V_{max} is observed in the hippocampus of these mice. However, in the SERT S44A/S48A Knock In mice, this effect is absent in both brain regions. The loss of CP94253-induced changes in uptake kinetics in the Knock In mice, both in the striatum and hippocampus, reinforces the critical role of these phosphorylation sites in mediating the regulation of SERT function.

Therefore, the presence of these specific phosphorylation sites in SERT appears to be crucial for the 5-HT_{1B} receptor-GSK3ß mediated modulation of SERT activity and, consequently, 5-HT reuptake dynamics.

CURRICULUM VITA

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EXPLORING SEROTONIN 1B AUTORECEPTOR MEDIATED REGULATION OF SEROTONIN TRANSPORTER IN FEMALE MICE

Key Impact:

Expanded understanding of serotonin regulation mechanisms in female mice, contributing to knowledge of potential sex differences in neurotransmitter systems.

Investigated region-specific (striatum and hippocampus) serotonin uptake regulation, providing insights into localized neurotransmitter dynamics.

Process and Outcomes:

Mastered various laboratory techniques including cell culture, biochemical assays (Uptake assays, Western blotting, Biotinylation).

Developed proficiency in mouse brain anatomy, brain removal, and precise dissection of specific brain regions.

Conducted comparative analysis between male and female mice to identify potential sex-based differences in serotonin transporter regulation.

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- Birari P, **Jalihalkar S**, Gaykhe A, Mishal R, & Mishal H (2020). SELF-MEDICATION AMONG PHARMACY STUDENTS- A THREATENING TREND. *International Journal of Modern Pharmaceutical Research*, *4*(3), 126–130. https://ijmpronline.com/admin/assets/article_issue/1593499537.pdf

POSTER PRESENTATIONS

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2. **5th METRxPLORE Undergraduate Research Conference 2020** Organized by Bhujbal Knowledge City Institute of Pharmacy, Nasik.
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- Coursera course on **Clinical Trials: Good Clinical Practice Specialization Course** by Novartis**.**
- Coursera Course on **Understanding the Brain: The Neurobiology of Everyday Life** by Dr. Peggy Mason, University of Chicago.
- Coursera course on **Evidence Based Toxicology,** by Thomas Hartung and Lena Smirnova, Johns Hopkins University
- Registered Pharmacist in India

INTRODUCTION

MAJOR DEPRESSIVE DISORDER (MDD)

Depression, also known as Major Depressive Disorder (MDD) is a common yet perilous mood disorder affecting over 8.4% - 21 million people in the year 2020 in the United States. [1,2] It affects the individual's ability to feel, think and carry out daily activities normally. [2,3] It induces a range of symptoms, that present as various physiological and psychological manifestations: [3]

1. Dysregulated Mood: Characterized by sadness, irritability, hopelessness.
2. Social Isolation and Decreased interest in previously enjoyed activities.
3. Sleep Disturbances: Ranging from insomnia to hypersomnia.
4. Persistent fatigue or loss of energy.
5. Unexpected fluctuations in weight or changes in appetite.
6. Psychomotor Retardation or Agitation.
7. Cognitive Impairment: Difficulty concentrating or impaired memory.
8. Physiological Symptoms: Headaches, Gastrointestinal (GI) disturbances, Unexplained Pain.
9. Suicidal Ideation: Persistent or recurrent suicidal thoughts.

The etiology of MDD is multifaceted and complex. It involves a multitude of factors including genetics and epigenetics[4,5] along with environmental impact caused due to socioeconomic deprivation, traumatic incidents and childhood abuse/

neglect and the psychological factors manifested into cognitive impairments and biochemical or molecular mechanisms that involve the monoamine pathways or inflammatory responses.[5] Additionally, the existence of MDD has been found to be associated with an increased risk of not only developing but also exacerbating several comorbidities including Central Nervous System (CNS) disorders, Cardiovascular (CVS) diseases, diabetes, obesity, autoimmune diseases, and substance use disorder further complicating our understanding of MDD.[6]

CONTEMPORARY APPROACHES TO MDD MANAGEMENT

Pharmacological Interventions targeting the monoamine hypothesis

The monoamine hypothesis, which has been around for over 50 years now, suggests that individuals who struggle with depression have diminished levels of the monoamines like the serotonin (5-HT), norepinephrine (NE) and dopamine (DA). [7–11] This was determined using various theories, including:

1. The action of lysergic acid diethylamide (LSD), that it interferes with the peripheral 5-HT receptors by blocking it. [7]
2. The use of reserpine as an antihypertensive, which results in decreased levels of vesicular monoamine stores and increased levels of the 5-HT metabolite 5-HIAA; and followed by the use of DOPA, a precursor of NE, which reversed these effects caused by reserpine. [7,12,13]
3. The elevation of mood after using iproniazid, an antimicrobial drug that was used in the treatment of TB patients, later emerged to be a monoamine oxidase inhibitor. [7,14]
4. Studies manipulating dietary tryptophan levels, which showed that tryptophan depletion can lower mood in some individuals, while tryptophan supplementation may have antidepressant effects in certain patients. [15]

These hypotheses collectively led to the development of an array of antidepressants that now include tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs), selective norepinephrine reuptake inhibitors (SNRIs), and monoamine oxidase inhibitors (MAO-I). [16] However regardless of the advancement of a variety of antidepressant options, notable drawbacks persist. For example, despite the fact that SSRIs are the first line of treatment, many patients either do not respond to them effectively or lack the ability to tolerate their different side effects including nausea, weight gain, sexual dysfunction, or insomnia which can impair their quality of life.[17] In addition, research has also shown that it may take up to 6–8 weeks to see the desired results, and even then, 60% of patients have residual impairments from the treatment that increases the likelihood of recurrence of MDD. [17–20] Moreover, some patients may not respond well to any antidepressants currently on the market, which results in depression resistant to treatment.[21] It's important to note that there are significant sex based differences in both the etiology and treatment responses of MDD like women being approximately twice as likely as men to develop MDD. [22,23] Some studies also suggest that women may respond better to SSRIs, while men may show a better response to TCAs. [22] These differences could be due to variations in brain structure, neurotransmitter systems, and hormonal influences between males and females. However, research findings in this area have been mixed and more

studies are needed to fully understand the impact of sex on MDD treatment outcomes. These drawbacks emphasize the necessity of an alternative or adjunctive therapy to more effectively and thoroughly address the complexities associated with MDD.

OVERVIEW OF THE SEROTONERGIC SYSTEM

Serotonin, also referred to as 5-Hydroxytryptamine (5-HT), is an extremely prevalent monoamine neurotransmitter that was isolated and identified by Maurice Rapport and Irvine Page in 1948. This monoamine neurotransmitter is produced mainly by the enterochromaffin cells of the gastrointestinal tract, as well as by serotonergic neurons in the central nervous system, and in platelets. [24,25,26]

LOCALIZATION OF THE SEROTONERGIC SYSTEM

1.a Human

1.b Rodent Brain

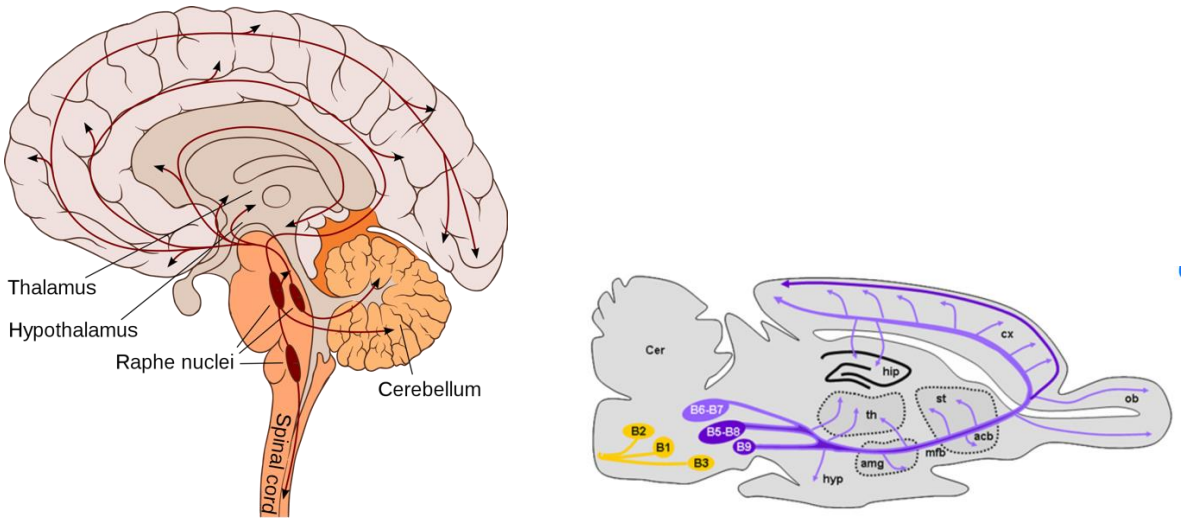


Figure 1. Innervations of the Serotonergic System in a. human and b. rodent brain

Dalhöstrom and Fuxe were the first to anatomically locate serotonergic pathways in the central nervous system using histochemical fluorescence techniques by mapping these cell bodies within the brain stem's raphe nuclei of rodents.[27] The raphe nuclei comprises of heterogeneous populations of neurons with distinct

morphologies, projections, and neurochemical characteristics in both animals and humans. It is divided into the dorsal raphe nucleus (DRN) that projects to the striatum, amygdala, and the prefrontal cortex, the median raphe nucleus (MRN) heavily innervating the hippocampus and septum, and the caudal raphe nuclei, all of which play a crucial role in the brain's serotonergic system. [28–31]

Having explored the localization of the serotonergic system, we now proceed to look at the synthesis, storage, release and reuptake of 5-HT, which are pivotal processes in understanding the regulation and function of this neurotransmitter.

SYNTHESIS, STORAGE, RELEASE AND REUPTAKE

Tryptophan is a crucial naturally occurring amino acid necessary for the synthesis of 5-HT. It is converted to 5-hydroxy-L-tryptophan by the enzyme tryptophan hydroxylase I at the serotonergic neuron, which is also the rate-limiting step in the synthesis of 5-HT. Subsequently, 5-hydroxytrptamine gets rapidly decarboxylated into 5-HT by the enzyme L-amino acid decarboxylase, and then 5-HT is rapidly transported into the vesicles by the vesicular monoamine transporters. [25,32]

Exocytosis of these vesicles releases 5-HT into the synaptic cleft in a TTX-sensitive and Ca^{2+} dependent manner. The reuptake into the presynaptic terminal is via a Na^{+}/Cl^{-}dependent 5-HT transporter (SERT) that regulates the availability of extra-neuronal 5-HT for 5-HT receptors and subsequent serotonergic neurotransmission.

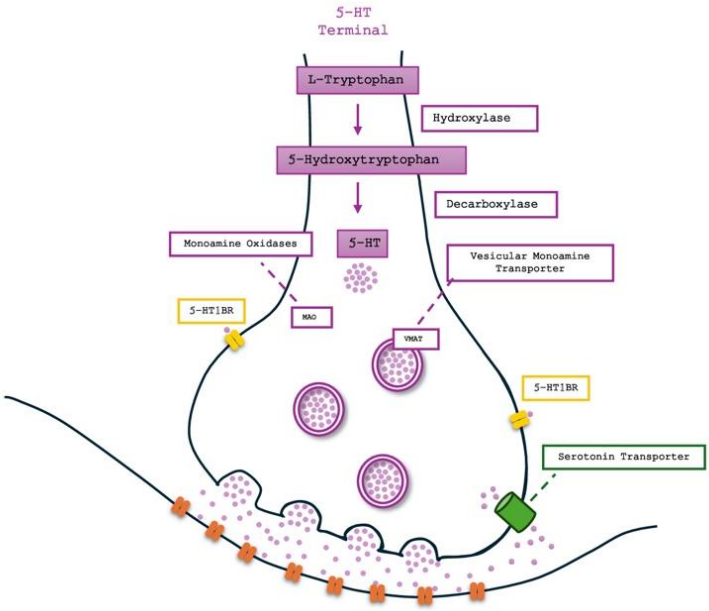


Figure 2. Synthesis, Storage, Release and Reuptake; image created using BioRender.

Along with that the enzymes monoamine oxidase and aldehyde dehydrogenase are also essential in the breakdown of 5-HT into its inactive form, 5-hydroxyindole acetic acid. [26]

IMPORTANCE OF THE SEROTONERGIC SYSTEM

5-HT is a multifaceted neurotransmitter that is essential for modulating a variety of physiological and psychological processes. It affects a variety of activities, such as appetite, mood, motor function, cognitive, and autonomic processes.[24–26] Changes in 5-HT levels have been linked to mood disorders like depression, anxiety, obsessive compulsive disorder, eating disorders and schizophrenia.[25,26] Additionally, 5-HT plays a physiological role in neuroendocrine processes such as controlling the pituitary gland's release of hormones, modulating circadian rhythms by acting on the suprachiasmatic nucleus, regulating eating habits and satiety, and serving as a precursor to the hormone melatonin, which controls seasonal reproductive cycles and sleep-wake cycles.[33] Moreover, different states of consciousness, such as peaceful waking states and rapid eye movement (REM) sleep, have also been shown to be correlated with varied firing patterns of the serotonergic neurons.[26] Furthermore, 5-HT plays a crucial role in modulating brain activity and behavioral arousal. It establishes the baseline level of neural activation, influencing overall alertness and responsiveness of an individual. When 5-HT receptors are activated, they enhance both cognitive and motor functions, promoting increased arousal and motor activity. Additionally, 5-HT activation inhibits the processing of irrelevant sensory information.[25,34]

Overall, 5-HT plays a complex and pivotal role in regulating psychological states, behavioral responses, and physiological processes throughout the body.

COMORBIDITY AND COGNITIVE IMPAIRMENTS IN MAJOR DEPRESSIVE DISORDER (MDD) IN RELATION TO SEROTONIN

Given serotonin's extensive role in regulating mood and cognitive functions, its involvement in Major Depressive Disorder (MDD) and associated cognitive impairments becomes particularly significant. MDD frequently coexists with anxiety disorders which complicates the treatment outcomes exacerbating cognitive impairments associated with memory and attention. Numerous studies have highlighted the bidirectional relationship between depression and anxiety, affecting and making it difficult for individuals to concentrate, remember information and make decisions.

Chronic stress is also recognized as one of the primary etiological factor in the development of Major Depressive Disorder (MDD), with impaired memory being a significant diagnostic symptom of this condition[35,36]. There has also been evidence showing reduction in hippocampal volumes in depressed adults, showing that the hippocampus plays a key role in depression. There have also been a lot of evidence of such striatal abnormalities.[37]

5-HT pathways, receptors, and transporters, which are extensively present in brain regions critical for learning and memory, have been shown to play a crucial role in this intersection of memory impairment and Major Depressive Disorder (MDD)[35]. Studies have also indicated that pharmacological or genetic manipulation of these

5-HT receptors and transporters can modulate learning and memory processes and depletion of 5-HT has been shown to impair memory in both animal models and human subjects.[38–40] Neuroimaging studies have substantiated these findings, showing that manipulating 5-HT levels does modulate activity in these brain regions associated with memory and emotional processing.[41]

Despite all the advancements, there still exists gaps in understanding the precise mechanism by which 5-HT influences memory in the context of depression. There is a need to better understand how individual variations in the serotonergic system can contribute to the memory impairments seen in depression.

5-HT RECEPTORS

As previously indicated, 5-HT induces an extensive spectrum of physiological effects in humans. These effects are mediated through 15 distinct receptor subtypes, all of which are G protein coupled receptors (GPCRs) except for one ligand-gated receptor. [42,43] Over 700–800 million years, 5-HT receptors have evolved considerably, and the current classification scheme identifies seven different types of 5-HT receptors, 5HT1–7. The structure of these receptors is characterized by seven transmembrane domains, an intracellular carboxy-terminus, and an extracellular amino-terminus that allows the receptors to interact with G proteins, enabling them to modulate various effector systems such as ion channels, phospholipase C, and adenylyl cyclase. 5-HT- GPCRs can influence numerous biochemical signaling pathways with multiple physiological ramifications by coupling to Gαi, Gαq/11, and Gαs in all three conventional signaling pathways, with the only exception as previously mentioned being the 5-HT3 receptor which is a ligand-gated ion channel receptor.[44]

The 5-HT1 receptor subtypes—HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, and 5-HT_{1F} and the $5HT_{5A}$ and $5HT_{5B}$—are Gi-coupled and they act by inhibiting adenylate cyclase, and subsequently diminishing the production of cAMP. The 5-HT2 receptors - 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} are Gq/11-coupled receptor that activates phospholipase C, leading to an increase in the production of inositol trisphosphate and

diacylglycerol which further initiates a downstream signaling cascade. The remaining excitatory metabotropic 5-HT receptors - 5-HT4, 5-HT6, and 5-HT7, are mediated by Gs-coupling leading to adenylate cyclase activation, and enhanced cAMP production.[44,45]

While all 5-HT receptors can be potently activated by serotonin, each subtype exhibits unique characteristics in terms of signal-transduction mechanisms, neuroanatomical distribution, and affinities. These differences create opportunities for drug discovery, making each 5-HT receptor subtype a potential therapeutic target. This diversity in receptor properties and functions emphasizes on the complexity of the serotonergic system and its wide-ranging effects on human physiology.

FOCUSING ON THE IMPORTANCE OF RECEPTOR- 5HT$_{1B}$

The 5-HT$_{1B}$ receptors, as mentioned previously, are Gi protein coupled. [43,44,46] They have a putative seven transmembrane spanning structure. The gene coding for the mouse 5-HT$_{1B}$ receptor is located on chromosome 9 (9E) and in humans located on chromosome 6 (6q13). [47–49] The amino acid sequence of this receptor to a high degree is similar for humans and rodents with almost around 93% homology. [47] 5-HT$_{1B}$ receptor has been shown to be involved in a very broad range of physiologic effects including satiety, sleep, locomotor activity, social interaction, sexual behavior and ejaculatory function, reduction of body temperature, modulation of memory and learning.

Multiple pharmacological investigations have indicated that 5-HT$_{1B}$ receptors are present in both serotonergic and non-serotonergic neurons, serving as autoreceptors [48,50–54] and heteroreceptors.[47,48,55] The 5-HT$_{1B}$ autoreceptors play a role in regulation of 5-HT release. Upon binding to the 5-HT$_{1B}$ receptors, 5-HT inhibits the formation of cAMP leading to downstream cellular responses and diminished transmitter release whereas the 5-HT$_{1B}$ heteroreceptors modulate the activity of the release of neurotransmitters like glutamate, GABA, acetylcholine and dopamine.[47] According to Bruinvels, A.T, in the rat brain, the 5-HT$_{1B}$ receptor mRNA displayed an extensive yet unique distribution pattern. High levels of expression were found in the CA1 region of the hippocampus, Purkinje cells of the

cerebellum, olfactory tubercle, subthalamic nucleus, and medium-sized spiny neurons of the caudate-putamen, while nucleus accumbens, several cortical regions, and a few thalamic and hypothalamus nuclei have all shown moderate expression of the 5HT1B mRNA and the posterior communicating artery and other small cerebral arteries were shown to harbor 5-HT_{1B} mRNA, indicating a possible function of the 5-HT_{1B} receptor in the regulation of cerebrovascular health.[56] Overall, 5-HT_{1B} mRNA's widespread yet distinct expression pattern suggests that this receptor subtype has a variety of uses in the central nervous system.

In a study by Montañez et al, the absence of 5-HT_{1B} autoreceptors in KO mice resulted in an antidepressant-like phenotype, with reduced anxiety-like and depressive-like behaviors. Similarly, it has also been shown that increased extracellular 5-HT levels in the ventral hippocampus suggest a role for these autoreceptors in regulating 5-HT release. These findings together highlight the potential of targeting 5-HT_{1B} autoreceptor signaling for the treatment of anxiety and depression.

Given the multifaceted roles of the 5-HT_{1B} receptor, it emerges as a crucial element in understanding the pathophysiology of these mental health disorders and targeting this 5-HT_{1B} receptors and the signaling pathways that it affects could offer novel therapeutic strategies, providing a promising avenue for future research and clinical application in mood disorders like MDD, anxiety disorders and ASD.

5-HT TRANSPORTER (SERT)

Although, there are more than 15 subtypes of the 5-HT receptors, the termination, or the clearance of the extracellular 5-HT from the synaptic cleft is carried out by a single gene (*SLC64A* for solute carrier family 6, member A4)[57]-encoded protein namely the 5-HT transporter (SERT), expressed on the presynaptic 5-HT neuronal axon terminals. SERT transports 5-HT which is dependent on Na^+/Cl^-.[58,59,60] Apart from the expression of SERT in 5-HT neurons in CNS to reuptake of released 5-HT into neurons, the SERT is also expressed in peripheral nervous system regulating 5-HT uptake by vascular smooth muscle cells, endothelial cells, placenta and platelets. This transporter, along with the DAT, NET, and GABA Transporters, is a member of the extensive neurotransmitter sodium symporter family (NSS).[59,60] The Human 5-HT transporter (hSERT), as first cloned by Dr. Sammanda Ramamoorthy, encodes 630 amino acids with 12 putative Transmembrane domains with intracellular cytoplasmic NH_2 and COOH terminals (Figure 3) and has been localized in the chromosome 17q11.2 in humans.[58,61] SERT amino acid sequence revealed several putative canonical phosphorylation sites for specific protein kinase mediated regulation of SERT function, trafficking and stability.

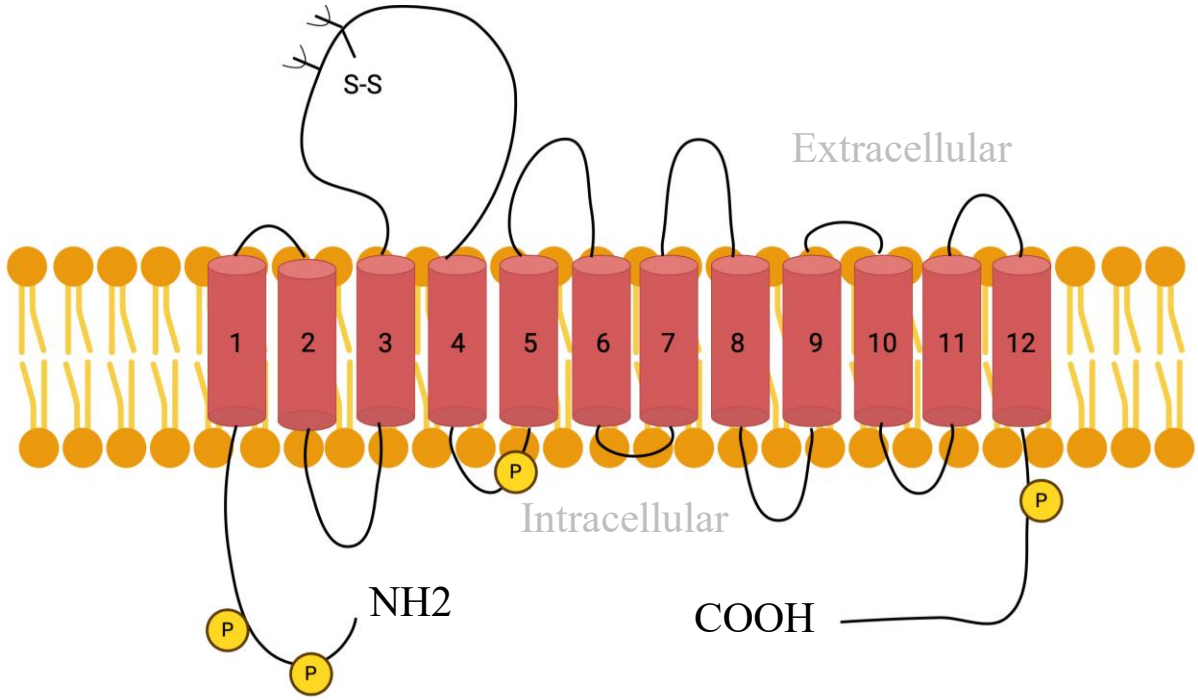


Figure 3. Diagrammatic representation of Serotonin Transporter; Created using BioRender

REGULATION OF SERT:

The modulation of the SERT function is significantly influenced by the presynaptic kinases and phosphatases (Table.1). Depletion of intracellular Ca^{2+}, inhibition of calmodulin, CaMKII, Src-kinase, p38 MAPK, and activation of PKC all result in an immediate decrease of SERT function and conversely, SERT activity can be stimulated by elevated intracellular Ca2+, NOS/cGMP activation, and MAPK pathway activation.[57,58] There have also been studies showing the interdependent relation between the phosphorylation of the transporter to the transport activity and the surface expression of the transporter, like for example in the PKC dependent

pathway, phosphorylation of SERT internalizes the transporter, reducing the cell surface expression and thus reducing the 5-HT uptake. [57,58,62]

The table below highlights some of the key regulators of SERT [58,63–69,71–77]

Regulators	Effects on SERT
Protein kinases	
PKC	Activation: Decreased SERT Vmax and surface SERT. Increased SERT endocytosis and phosphorylation[63–68]
p38 MAPK	Inhibition: Decreased SERT Vmax and Km. Decreased SERT basal phosphorylation with or without changes in surface SERT proteins.[65,69,70]
PKG	Activation: Decreased SERT Vmax and surface SERT. Increased SERT endocytosis and phosphorylation[67,71]
CaMKII	Inhibition: Decreased 5-HT uptake[72]
Tyrosine kinase	Inhibition: Decreased 5-HT-uptake and Vmax[73,74]

GSK3α/β	Inhibition: Increased 5-HT-uptake and Vmax. Increased surface SERT levels and decreased SERT phosphorylation.
Phosphatases	
PP2A	Inhibition: Decreased 5-HT uptake and increased SERT phosphorylation. Associates with SERT.[68,75]

Table 1. Protein Kinases and Phosphatases that regulate SERT.
Table adapted from: Ramamoorthy S, Shippenberg TS, Jayanthi LD. Regulation of monoamine transporter: Role of transporter phosphorylation. Pharmacol Ther. 2011 Feb;129(2):220-38.

In addition to these key regulators, various presynaptic auto- and hetero-receptors play crucial roles in modulating SERT activity. The 5-HT_{1B} receptor, located on presynaptic terminals, is a well-established regulator of SERT function. However, other receptor subtypes also influence 5-HT reuptake. For instance, 5-HT_{1A} receptors, distributed on both serotonergic neurons and target cells, can modulate 5-HT release and indirectly impact SERT function, the 5-HT4 receptors have been demonstrated to upregulate BDNF levels, potentially influencing SERT expression or activity and the 5-HT_{2C} receptors may also contribute to 5-HT level regulation, though their precise effect on SERT remains to be fully elucidated.[78] In addition to serotonin receptors, other receptors like histamine receptors (H3R) and

BDNF/TrkB increase 5-HT uptake, while α2 adrenergic receptor activation decreases 5-HT uptake.[79–82]

Owing to the significance of SERT in preserving the serotonergic tone, and neurotransmission, dysregulation of SERT has been implicated in mood disorders like MDD, anxiety, obsessive compulsive disorder (OCD), autism spectrum disorder (ASD), and attention deficit hyperactive disorder (ADHD). Additionally, the existence of a short chain allele, a variant in the SERT promoter has been linked to psychiatric conditions related to SERT function.[78] Due to its significant influence on 5-HT reuptake and overall neurotransmitter balance, targeting SERT remains a primary treatment strategy like the SSRIs/SNRIs for MDD and other related disorders. Continued research into SERT regulation and its genetic variants offers promising avenues for developing more effective and personalized therapeutic interventions for these conditions.

INTERACTIONS BETWEEN 5-HT$_{1B}$ AND SERT

Both 5HT$_{1B}$ and SERT are localized on the presynaptic terminals of the serotonergic neurons, where they play critical roles in the autoregulation of the serotonergic neurotransmission. When activated, 5HT$_{1B}$ autoreceptors inhibit the release of 5-HT in the synaptic cleft through a negative feedback mechanism. Concurrently, SERT functions to clear 5-HT from the synaptic cleft via high affinity reuptake. Alterations in signaling mediated by either 5-HT$_{1B}$ or SERT expression and function have been implicated in the serotonergic dysfunction, particularly emotional regulation.[83–85] Understanding the intricate interplay between these two components could provide valuable insights into the modulation of serotonergic signaling.

This has been supported by data from studies using 5-HT$_{1B}$ and SERT knockouts. It has been demonstrated that the modulation of extracellular 5-HT levels involves both SERT and 5-HT$_{1B}$ autoreceptors. These studies used cyanopindolol a 5-HT$_{1B}$ antagonist, and in order for the 5-HT$_{1B}$ antagonist cyanopindolol to block 5-HT clearance in the CA3 region of the hippocampal region, both the 5-HT$_{1B}$ receptor and SERT are required.[80,81,86] When either of these crucial serotonergic neurotransmission-regulating proteins is constitutively knocked down, cyanopindolol's capacity to limit 5-HT clearance is eliminated. These findings

contribute to the increasing body of research indicating that 5-HT_{1B} receptors regulate SERT.[80,81]

In a more recent paper, genetic deletion or pharmacological blockage of 5-HT_{1B} autoreceptors has shown to impair the function of the 5-HT transporter (SERT) in synaptosomes and moreover, administration of a selective 5-HT_{1B} agonist or by increasing 5-HT_{1B} autoreceptor expression using viral-mediated gene transfer has shown to upregulate the expression of 5-HT_{1B} autoreceptor, consequently improving SERT activity.[87]

The interplay between 5-HT_{1B} autoreceptors and SERT emphasizes on the importance of these mechanisms in modulating serotonergic signaling. Understanding the regulation of 5-HT clearance by 5-HT_{1B} receptors and SERT elucidates the complexity of serotonergic neurotransmission.

WHAT IS GSK3 α/β AND ITS RELEVANCE TO 5-HT$_{1B}$ MEDIATED SERT REGULATION

GSK3 α/β is a constitutively active serine/threonine kinase and has two isoforms GSK3α and GSK3β.[88–90] The dysregulation of GSK3 α/β has been associated with mood disorders, neurodegenerative disorders like alzheimer's and parkinson's, diabetes, inflammation, cancer.[89,91–93] Owing to the growing interest due its implication in various conditions, GSK3 α/β regulation is an important aspect that needs to be further investigated.

According to Varman et al., pharmacological inhibition of GSK3 α/β in mouse striatal synaptosomes, upregulated SERT activity, surface SERT levels and apparent affinity (K_m) in a phosphorylation dependent manner, indicating a regulatory role of GSK3 α/β in SERT function.[94] Out of the two isoforms, the intricate relationship between GSK3β and SERT is further highlighted by studies on GSK3β Knock In mice, which exhibit hyperactive and depression-like behaviors, suggesting a link between GSK3β activity and serotonergic function.[88,91,95,96] The amino acid sequence analysis has identified potential serine/threonine GSK3β phosphorylation sites within the consensus sequence motif S/T-X-X-X-S/T(P) within the N-terminal region of human SERT, suggesting a mechanism by which GSK3β may regulate SERT function through phosphorylation.[89,94] The S44 and S48 sites in the cytoplasmic N-terminal of SERT

are the two canonical sites of GSK3α/β based phosphorylation on SERT.[94] To further elucidate their roles in GSK3α/β mediated regulation, serine residues at the positions 44 and 48 were individually substituted with alanine (hSERT-S44A/S48A) to prevent phosphorylation of SERT and enhance SERT activity and surface expression on inhibition of GSK3β, establishing that these sites S44/48 are important for phosphorylation and regulation of SERT through GSK3β regulation.[94]

In conclusion, these findings highlighted the intricate regulatory role of GSK3β in modulating SERT function and surface expression, suggesting phosphorylation of these sites by GSK3β likely triggers a cascade of intracellular events leading to decreased SERT activity. However, how and which presynaptic receptor(s) on serotonergic neurons mediate the regulation of GSK3ß and subsequently SERT regulation is still unknown.

Recent studies have revealed an intricate relation that exists between 5-HT_{1B} and GSK3β. Using bioluminescence resonance energy transfer (BRET) assays followed by co-immunoprecipitation, a direct association has been observed in cultured heterologous cells.[97,98] GSK3β has been shown to modulate 5-HT_{1B} receptor function, particularly its coupling to Gi proteins and downstream signaling pathways, including activation of Akt.[99–101] Interestingly, primary unpublished data

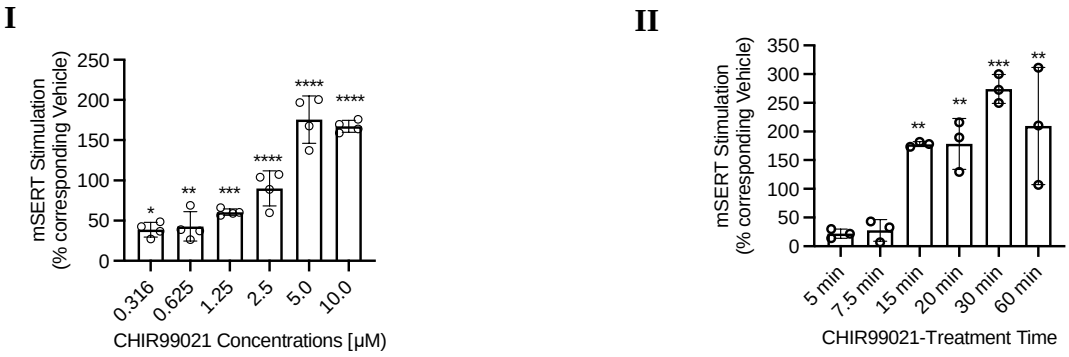
suggests that 5-HT_{1B} receptor activation may influence regulation of GSK3β through phosphorylation. This might present as a complex scenario as, 5-HT_{1B} autoreceptor activation typically inhibits 5-HT release, while increased 5-HT levels are associated with increased GSK3β phosphorylation.[102] Thus, it raises a possibility that 5-HT_{1B} autoreceptor mediated inhibition of GSK3ß via GSK3β phosphorylation might modulate not only 5-HT release but also increase 5-HT clearance through inhibiting SERT phosphorylation and activating of SERT, both of which are crucial factors for normal serotonergic neurotransmission and 5-HT linked physiological and behavioral functions. Dysregulation of this synchronized normal 5HT release and clearance leads to psychiatric disorders and comorbid disorders underlying psychiatric disorders. It further underscores the signaling complexity of serotonergic regulation and sheds light on potential therapeutic novel targets for neurological disorders involving serotonergic dysfunction.

RATIONALE

INTERPLAY BETWEEN 5HT1B, SERT, GSK3α/β

Having reviewed the various components and their interactions up to this point, lets now explore the rationale behind this project. To build up to this, we will take a closer look at how we reached this point by examining both previous published and unpublished data from our lab, which will help in understanding the context behind this project.

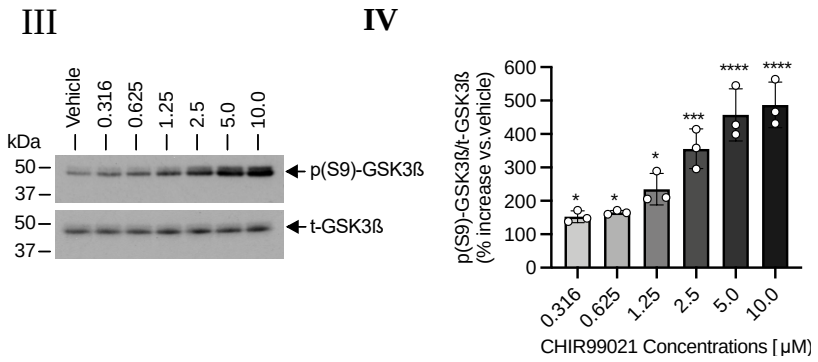
To provide a basic understanding of this project, our lab has previously established a connection between SERT and GSK3α/β. As shown in Panels I and II, treatment of mouse striatal synaptosomes with CHIR99021 (a GSK3α/β inhibitor) upregulates SERT-mediated 5-HT uptake in a dose- and time-dependent manner.



I, II. CHIR99021 pretreatment stimulates SERT activity in a dose and time dependent manner.

Figure obtained from: Ragu Varman D, Jayanthi LD, Ramamoorthy S. Glycogen synthase kinase-3β supports serotonin transporter function and trafficking in a phosphorylation-dependent manner. J Neurochem. 2021 Feb;156(4):445-464. doi: 10.1111/jnc.15152. Epub 2020 Sep 7. PMID: 32797733; PMCID: PMC7882002

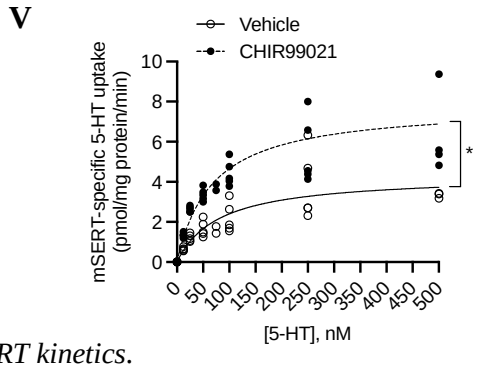
Concurrently, CHIR99021 treatment increases the inhibitory phospho(S9)-GSK3β without altering the total GSK3β levels, as demonstrated in Panel III and quantified in panel IV.



III, IV A dose dependent effect of CHIR99021 on t-GSK3β and p(S9)-GSK3β levels.

Figure obtained from: Ragu Varman D, Jayanthi LD, Ramamoorthy S. Glycogen synthase kinase-3β supports serotonin transporter function and trafficking in a phosphorylation-dependent manner. J Neurochem. 2021 Feb;156(4):445-464. doi: 10.1111/jnc.15152. Epub 2020 Sep 7. PMID: 32797733; PMCID: PMC7882002

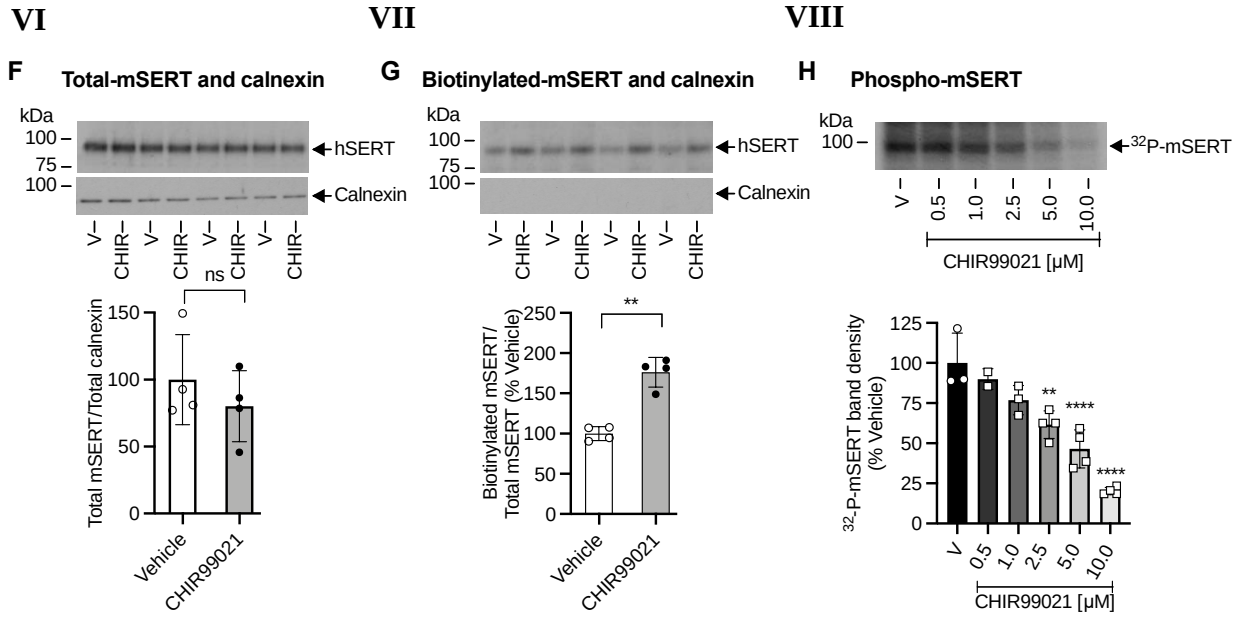
This increase in phospho(S9)-GSK3β can be used as an indicator of GSK3β activity inhibition due to phosphorylation. Next, panel V shows that blocking GSK3α/β in striatal synaptosomes increases the maximal velocity of 5-HT uptake with CHIR99021 treatment.



V. Effect of CHIR99021 on SERT kinetics.

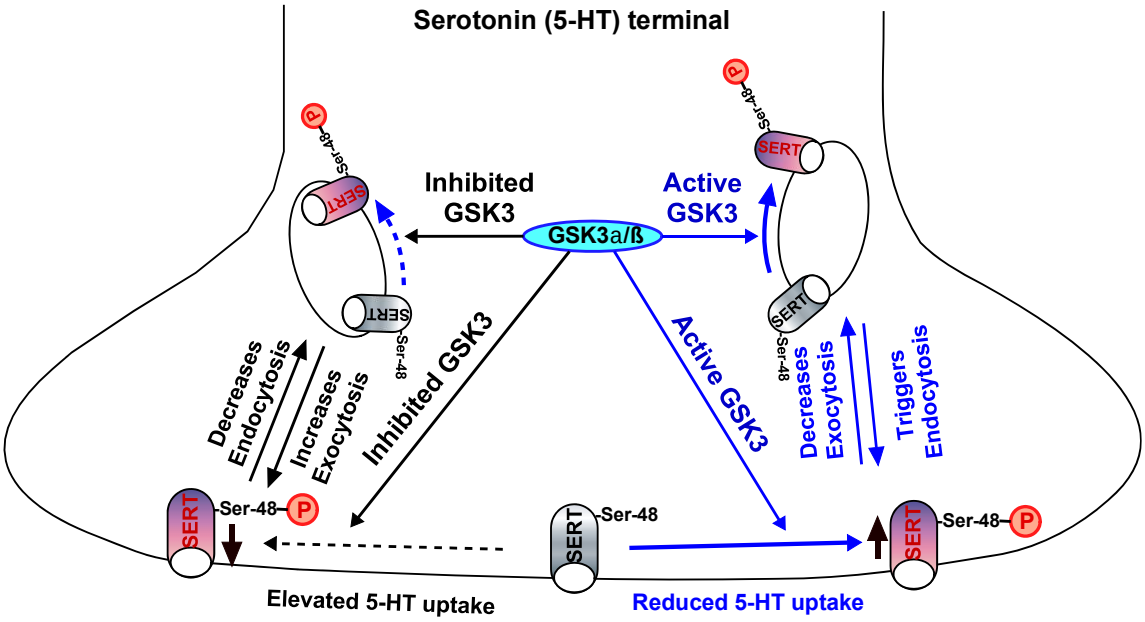
Figure obtained from: Ragu Varman D, Jayanthi LD, Ramamoorthy S. Glycogen synthase kinase-3β supports serotonin transporter function and trafficking in a phosphorylation-dependent manner. J Neurochem. 2021 Feb;156(4):445-464. doi: 10.1111/jnc.15152. Epub 2020 Sep 7. PMID: 32797733; PMCID: PMC7882002

To determine if this increase in maximal velocity was due to an increased presence of surface SERT, a biotinylation/immunoblot assay was performed. Panels VI and VII reveal that while CHIR99021 treatment did not affect the total level of SERT, it did increase the surface density of SERT in CHIR99021-treated synaptosomes. Since several kinases regulate SERT activity through phosphorylation, we investigated if GSK3α/β affects SERT through phosphorylation for which striatal synaptosomes were metabolically labeled with ^{32}P and treated with various concentrations of CHIR99021 and panel VIII shows that increasing concentrations of CHIR99021 decrease phosphorylated SERT in the synaptosomes.



VI, VII, VIII. Effect of CHIR99021 on surface SERT and effect of CHIR99021 on SERT phosphorylation

Figure obtained from: Ragu Varman D, Jayanthi LD, Ramamoorthy S. Glycogen synthase kinase-3β supports serotonin transporter function and trafficking in a phosphorylation-dependent manner. J Neurochem. 2021 Feb;156(4):445-464. doi: 10.1111/jnc.15152. Epub 2020 Sep 7. PMID: 32797733; PMCID: PMC7882002



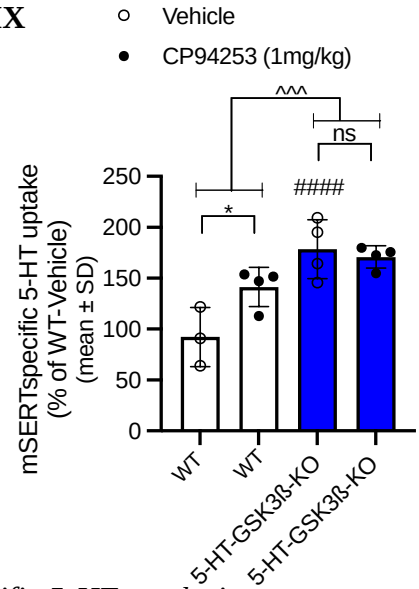
Diagrammatic representation of the complexity of Serotonergic Transmission involving GSK3β

Figure obtained from: Ragu Varman D, Jayanthi LD, Ramamoorthy S. Glycogen synthase kinase-3β supports serotonin transporter function and trafficking in a phosphorylation-dependent manner. J Neurochem. 2021 Feb;156(4):445-464. doi: 10.1111/jnc.15152. Epub 2020 Sep 7. PMID: 32797733; PMCID: PMC7882002

To summarize, this diagrammatic representation shows the complex interaction between GSK3α/β, SERT, and 5-HT release and uptake. The activity of GSK3α/β has a significant impact on SERT function. When GSK3α/β is blocked by CHIR99021, it leads to the phosphorylation and inhibition of GSK3α/β, which results in increased SERT uptake due to increase in surface SERT expression. And goes to show that active GSK3β phosphorylates SERT, which in turn decreases SERT mediated 5-HT uptake. Overall, this highlights the role of GSK3α/β in regulating SERT-mediated 5-HT uptake.

While this provides valuable insights into the role of GSK3α/β in SERT-mediated 5-HT uptake, it also raises questions about the specific effects of GSK3α/β on serotonergic function. To explore this further, a series of experiments currently unpublished was conducted previously by our lab (Durairaj Ragu Varman, Lankupalle D. Jayanthi and Sammanda Ramamoorthy 2023) focusing on the deletion of GSK3β specifically in serotonergic neurons. It was found that deleting GSK3β in these 5-HT neurons produced results like those observed with CHIR99021 treatment: the knockouts showed increased SERT uptake comparable to WT mice treated with CHIR99021. To determine if this effect was due to increased SERT surface expression, biotinylation and immunoblot assays were performed. These assays revealed that GSK3β knockouts had increased surface SERT levels like those in CHIR99021-treated mice, with no change in total SERT levels. Additionally, SERT phosphorylation studies showed that GSK3β knockouts had decreased phosphorylated SERT, mirroring the effects of CHIR99021. These findings collectively suggest that GSK3β is a crucial regulator of SERT function specifically within serotonergic neurons.

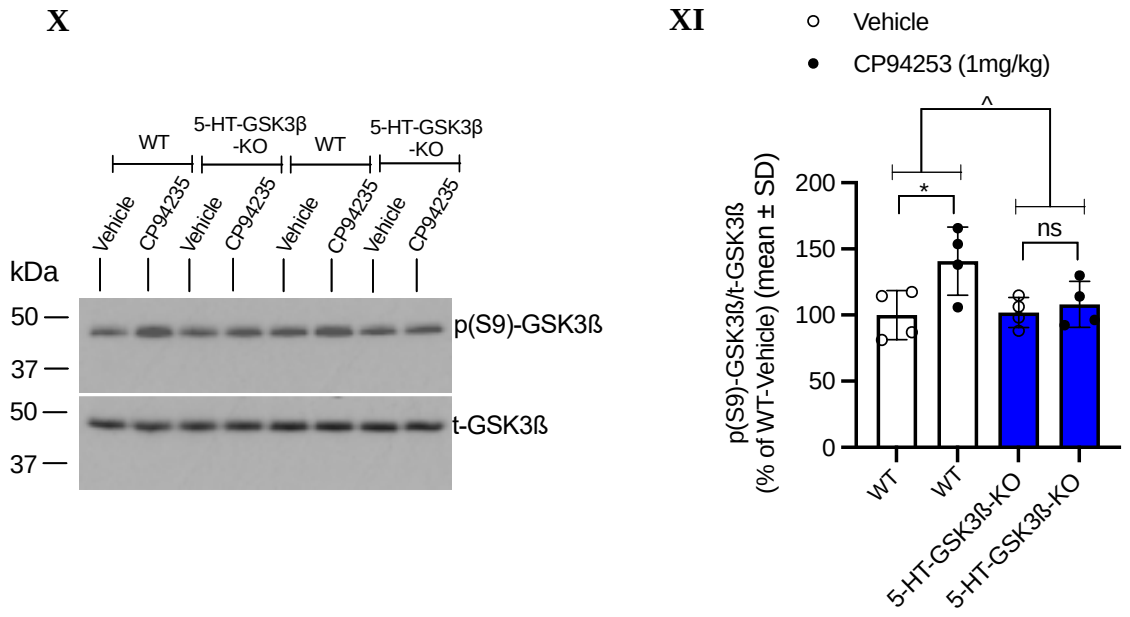
As previously discussed, the 5-HT_{1B} autoreceptor plays a role in SERT-mediated 5-HT uptake . There is also evidence suggesting that 5-HT_{1B} may affect the active/inactive states of GSK3β (Unpublished data by Durairaj Ragu Varman, Lankupalle D. Jayanthi and Sammanda Ramamoorthy 2023). This raises the possibility that the 5-HT_{1B} autoreceptor might mediate GSK3β inhibition through phosphorylation at the before mentioned consensus sequence motif S/T-X-X-X-S/T(P) within the N-terminal region of human SERT, which could influence 5-HT release and SERT-mediated uptake. To investigate this, our unpublished data show that treatment with CP-94253, a 5-HT_{1B} agonist, increases SERT-mediated 5-HT uptake in striatal synaptosomes. However, this effect is absent in 5-HT_{1B} agonist-treated GSK3β knockout mice, where SERT uptake is already elevated in both vehicle and treatment groups, as shown in panel IX.



IX. Figure showing the SERT specific 5-HT uptake in mouse synaptosome using Wild Type and 5-HT-GSK3β knock out mice

Unpublished data (Durairaj Ragu Varman, Lankupalle D. Jayanthi and Sammanda Ramamoorthy 2023)

Additionally, as demonstrated in panel X and XI phosphorylation studies using WT and KO mice treated with CP-94253 revealed that activation of 5-HT_{1B} by CP-94253 increased p(S9)-GSK3β levels in wild-type mice but did not alter GSK3β levels in knockout mice. This suggests that 5-HT_{1B} receptor activation inhibits GSK3β through phosphorylation, leading to enhanced SERT function.



X, XI. Figure showing the phosphorylation of GSK3β on activation of 5-HT_{1B} receptor using CP-94253.

Unpublished data (Durairaj Ragu Varman, Lankupalle D. Jayanthi and Sammanda Ramamoorthy)

Considering our current understanding, the interplay between 5-HT_{1B} receptors, SERT, and GSK3β suggests that the activation of 5-HT_{1B} autoreceptors on the presynaptic membrane not only inhibits the release of 5-HT but also promotes the phosphorylation and inactivation of GSK3β. This inactivation, in turn, enhances 5-HT uptake through the 5-HT transporter (SERT). Although the exact mechanisms are still under investigation, the primary rationale behind this project was to elucidate the complexities of this system in relation to depression, anxiety in female mouse models given the differences in how depression affects men and women, and the variations in treatment responses due to differences in the serotonergic system.[103]

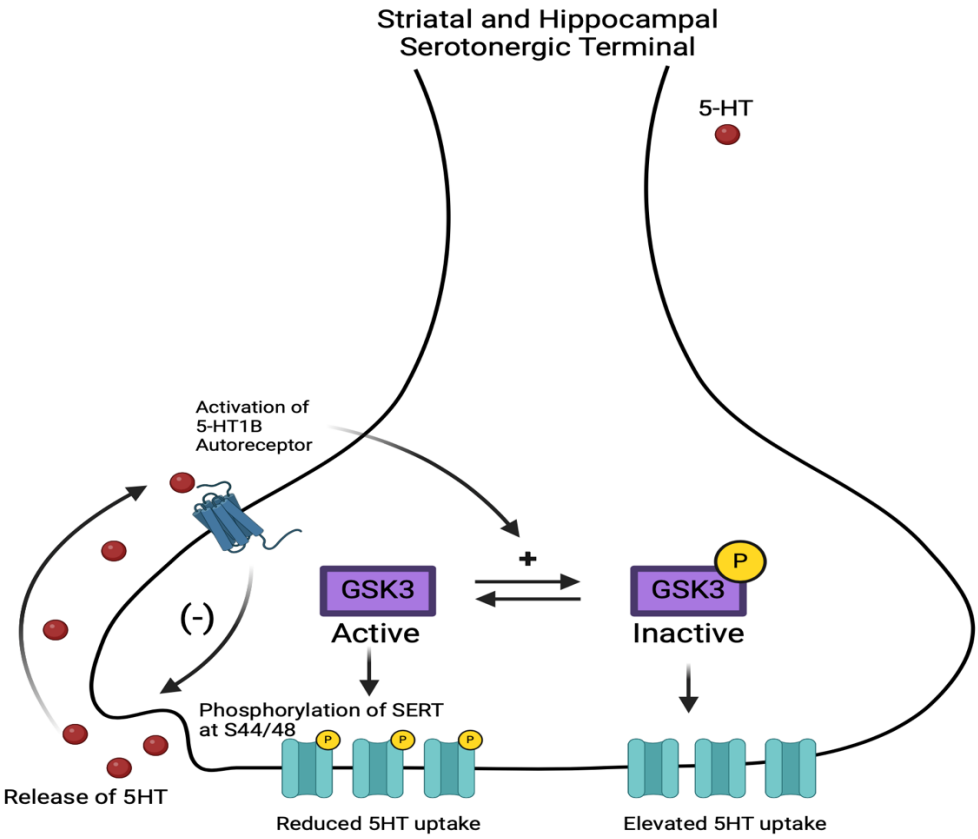


Figure 4. Diagrammatic representation of the rationale; Created using BioRender

The study aims to determine whether pharmacological intervention at the 5-HT_{1B} site alters uptake activity and the kinetics and cell surface expression of SERT through the S44A/S48A sites, similar to observations in male mice. Understanding these gender-specific mechanisms is crucial, as women not only exhibit differences in 5-HT binding sites in certain brain regions but also tend to have lower levels of SERT protein in nerve cells.[103] This project seeks to address these gender-specific aspects to improve our understanding and treatment strategies for depression and anxiety in women.

METHODS AND MATERIALS

Materials

All reagents and chemicals were purchased within 2 years and stored according to the manufacturer's instructions. CP-94253(5-Propoxy-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1*H*-pyrrolo[3,2-*b*]pyridine hydrochloride) (Cat# **1317**, Tocris), serotonin hydrochloride (Cat# H9523), Bradford protein assay (Cat# 5000006) were purchased from Bio-Rad (Hercules, CA), Fluoxetine ((±)-*N*-Methyl-γ-[4-(trifluoromethyl)phenoxy]benzenepropanamine hydrochloride, LY-110,140 hydrochloride) (Cat#PHR1394) All other chemicals were obtained from Tocris, Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Waltham, MA) unless otherwise indicated. Scruff Guards were used to minimize the stress on the animals during decapitation.

Animals

All animal procedures were followed in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and approved by Institutional Animal Care and Use Committee at Virginia Commonwealth University (approved protocol number AD10000476). Female adult wild-type and SERT -S44/48A Knock In mice (25-30 g body weight) were used. Both were C57BL/6J strain backgrounds. Mice were housed in a temperature- and humidity-controlled facility with a 12-h light/dark cycle at an ambient temperature of 19–22°C. All mice had free access to food and water throughout the study. Experiments were conducted during the light phase. Randomization was not performed to assign subjects. A total number of 34 female wild type and Knock In mice were used in this study, and the number of animals per experiment are described under figure legends. 5-HT Uptake experiments and Kinetic experiments were performed. Mice were subjected to rapid decapitation using the in-house protocol without prior anesthesia between 9:00 a.m. and 12:00 p.m. to obtain the brain. Animals were kept away from the area of sacrifice before decapitation to minimize stress and handling. They were proceeded as gently as possible to minimize the level of discomfort and pain to the animals.

Preparation of Crude Synaptosomes

In the brain, neurons communicate through computational units called synapses, which consist of pre- and post-synaptic terminals. Understanding the several processes that take place within the synaptic terminal is critical for understanding the pathophysiology of any disorder.[103]

Synaptosomes, a subcellular portion of closed synaptic connections isolated from brain tissue, contain all the cellular and molecular functions required for the storage, release, and reuptake of the various neurotransmitters.[104,105]

For the preparation of crude synaptosomes, unanesthetized mice were subjected to rapid decapitation, the brain was removed and dissected on ice. The striatum and the hippocampal region were removed and suspended in ice cold 0.32 M sucrose in 5 mM HEPES, pH 7.4 (sucrose buffer). The regions were then homogenized on ice and the homogenates were centrifuged at 1000 x g for 10 min at 4°C. When brain cells are gently homogenized, lipid bilayers reseal, creating subcellular fragments that contain synapses (terminals-synaptosomes).[105] The resultant supernatants were centrifuged at 12,000 x g for 20 min and the pellets containing the crude synaptosomes were resuspended in sucrose buffer. The protein concentration of each synaptosome preparation was determined by protein assay.

SERT functional assay (5-HT Uptake) in crude synaptosomes

5-HT uptake was performed as described by Samuvel et al. briefly, 30 µg of crude synaptosomes was incubated in KRH buffer, pH 7.4, (total volume : 300 ul containing (in mm): 25 mM Na_2HCO_3,124 mM NaCl, 5 mM KCl, 5 mM $MgSO_4$, 1.5 mM $CaCl_2$, 10 mM glucose, containing 0.1 mm ascorbic acid, 0.1 mm pargyline, and 10 nm [3H]5-HT for 5 min.[106] Crude synaptosomes were pre-incubated with the modulators at 37°C for 10 min followed by the addition of radiolabeled [^{3}H]5-HT to initiate 5-HT uptake. For saturation analysis, radiolabeled [^{3}H]5-HT was mixed with unlabeled 5-HT, ranging from 25 nM to 500 nM.

Nonspecific [^{3}H]5-HT uptake was defined as the accumulation in the presence of 0.1 µm fluoxetine (specific SERT inhibitor) and was subtracted from the total uptake. Uptake was terminated with the addition of 30 ul of stop buffer containing SERT inhibitor fluoxetine (0.1 µM) and kept on ice-cold slurry followed by rapid filtration over 0.3% polyethylenimine-coated glass fiber filters-B on a Brandel Cell Harvester. Filters were washed rapidly with 5 ml of cold PBS, and radioactivity bound to the filter was counted by a liquid scintillation counter. All uptake assays were performed in triplicate, and mean values of specific uptake from independent mice and each data point presented in the figures represents an individual mouse. The specific uptake ± standard deviation (SD) from all mice was determined.

Statistical analysis

Statistical analyses were done using GraphPad Prism software. All values are expressed as mean ± Standard deviation. Figures are presented in bar graphs showing every individual value representing a single experiment. For the uptake experiment, two-way ANOVA was followed by *post hoc* Bonferroni multiple comparisons. Student's t-test were used for kinetics and biotinylation studies. Statistical significance was considered at value of $P < 0.05$.

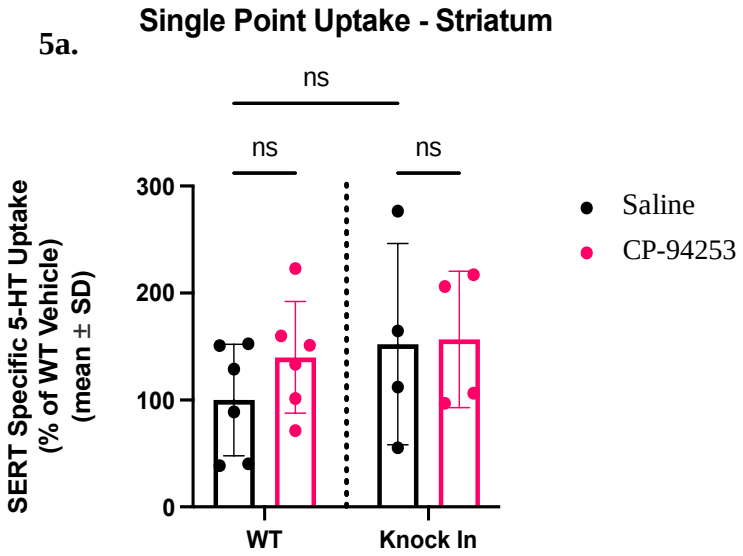
RESULTS

ACTIVATION OF 5-HT$_{1B}$ AUTORECEPTOR

Primarily, we examined if the GSK3α/β regulated phosphorylation sites Serine44 and Serine48 on SERT were involved in 5-HT$_{1B}$ autoreceptor mediated SERT regulation in female mice. We substituted Serine 44 and Serine 48 in SERT with non-phosphorylatable alanine using the CRISPR-Cas9 method to prevent phosphorylation of SERT by GSK3 α/β. The effect of 5-HT$_{1B}$ activation by the agonist CP-94253 was studied in wild-type (WT) females and SERT S44A/S48A Knock In female mice on 5-HT uptake.

In wild-type female mice, exposure to the 5-HT$_{1B}$ agonist CP-94253, given 30 minutes prior to decapitation, resulted in a trend towards increased SERT-mediated uptake in both the striatum and hippocampus regions. However, these increases were not statistically significant.

5a.



5b.

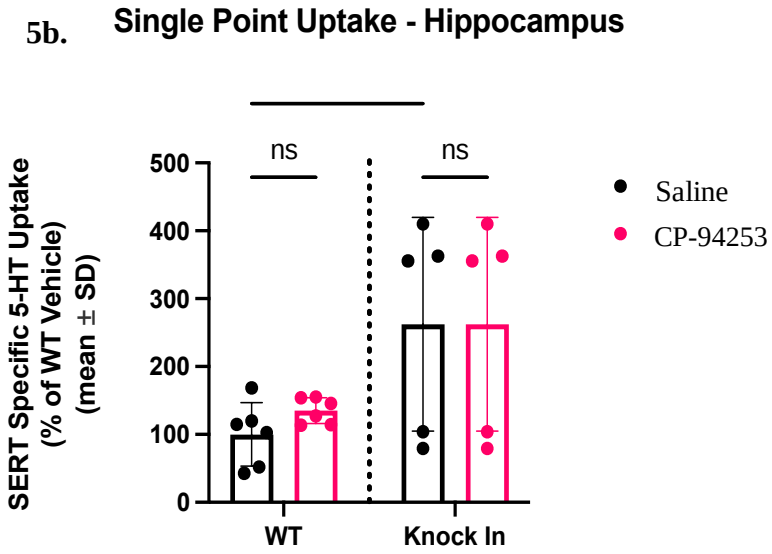


Figure 5a, b. Effects of CP-94253 on SERT- mediated 5-HT uptake in Female mice (a: Striatum (N=6); b: Hippocampus (N=5). Female mice (WT and Knock In) were injected with saline (shown in black) and drug (CP-94253 1mg/kg (shown in pink)) 30 mins prior to decapitation and dissection of the brain; 5-HT uptake was measured with 10nM 5-HT label. Uptake assays were executed in triplicates and the points show the average % uptake for each experiment. Analysis was done by 2-way ANOVA test. ns: non-significant
For striatum(Figure 5a), no significant effect of CP-94253 treatment was observed ($p > 0.05$). No significant effect of genotype was found ($p > 0.05$). There was no significant interaction between CP-94253 treatment and genotype ($p > 0.05$).
For the hippocampus (Figure 5b), a significant main effect of CP-94253 treatment was found ($p < 0.05$). No significant effect of genotype was observed ($p > 0.05$). No significant interaction between CP-94253 treatment and genotype was detected ($p > 0.05$).

In the SERT S44/48A Knock In (KI) females, both the vehicle (saline-injected) and CP-94253-treated groups showed a trend towards increased uptake compared to WT vehicle controls. However, there was no significant difference between the vehicle and CP-94253-treated KI groups. This suggests that substitution of serine to alanine in the KI animals may increase SERT uptake, possibly due to the inability of GSK3 α/β to phosphorylate SERT at the S44/48 sites. However, the activation of 5-HT_{1B} autoreceptors did not significantly further increase SERT uptake activity in either the striatum or hippocampus regions of the female KI mice.

These results differ from previously reported findings in male mice, where significant increases in SERT uptake were observed in both WT males treated with CP-94253 and in KI males regardless of treatment. This discrepancy suggests potential sex differences in the regulation of SERT through GSK3 α/β mediated by 5HT1B autoreceptor activation.

While our data show trends consistent with the involvement of Serine 44/48 sites in the regulation of SERT through GSK3 α/β mediated by 5HT1B autoreceptor activation, the lack of statistical significance in female mice prevents us from drawing firm conclusions. These results highlight the importance of considering

sex as a biological variable in neuropharmacological studies and suggest that the regulation of the 5-HT system may differ between males and females.

Further studies with larger sample sizes and consideration of factors such as estrous cycle may be necessary to fully elucidate the role of these phosphorylation sites in SERT regulation in females and to understand the extent of sex differences in this regulatory mechanism.

5-HT$_{1B}$ MEDIATED ALTERATIONS IN SERT KINETICS

Next Kinetic studies were done to study the rate and saturation of 5-HT Uptake under the influence of CP-94253 (5-HT$_{1B}$ Agonist (30 mins)). In wild type females, striatum treatment with CP-94253 resulted in a non-significant increase in the maximal velocity (Vmax) (t=1.171, df=4; *P*=0.3065) and non-significant changes in Km between vehicle and treatment (t=1.347, df=4; *P*=0.2494).

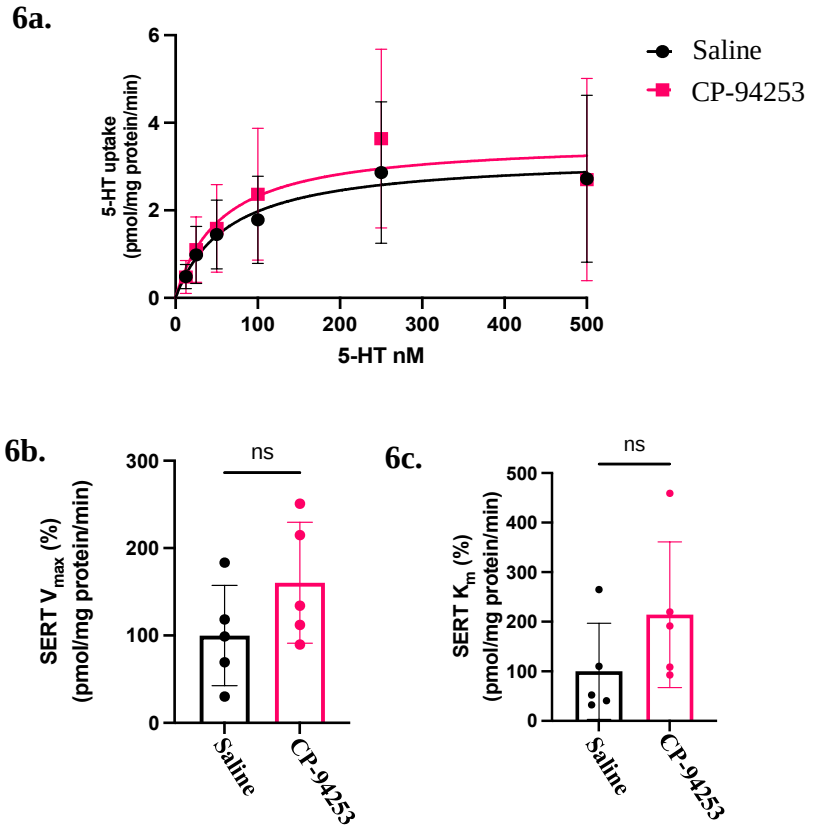


Figure 6a, b, c. Effects of CP-94253 on SERT Kinetics in Female mice (Wild Type Striatum N= 5). Wild-type female mice received injections of saline (shown in black) or CP-94253 at 1 mg/kg (shown in pink) 30 minutes before brain dissection. Serotonin (5-HT) kinetics assay was performed in triplicate, with data points representing the average for each concentration across all experiments. Non-linear curve fits of uptake data were generated for vehicle (N=5) and CP-94253-treated (N=5) groups. Statistical analysis was conducted using paired t-tests (*P < 0.05). Panel (a): Non-linear curve fits illustrating 5-HT uptake kinetics. Panel (b): Comparison of maximum uptake velocity (Vmax)
between vehicle and CP-94253-treated groups. Panel (c): Comparison of affinity (Km) for the serotonin transporter (SERT) under both conditions. ns - non-significant.

In S44A/S48A Knock In females, striatum treatment with CP-94253 showed no difference between the maximal velocity (V_{max}) (t=0.08725, df=2; *P*=0.9384) and non-significant changes in Km between vehicle and treatment (t=0.2535, df=2; *P*=0.8236).

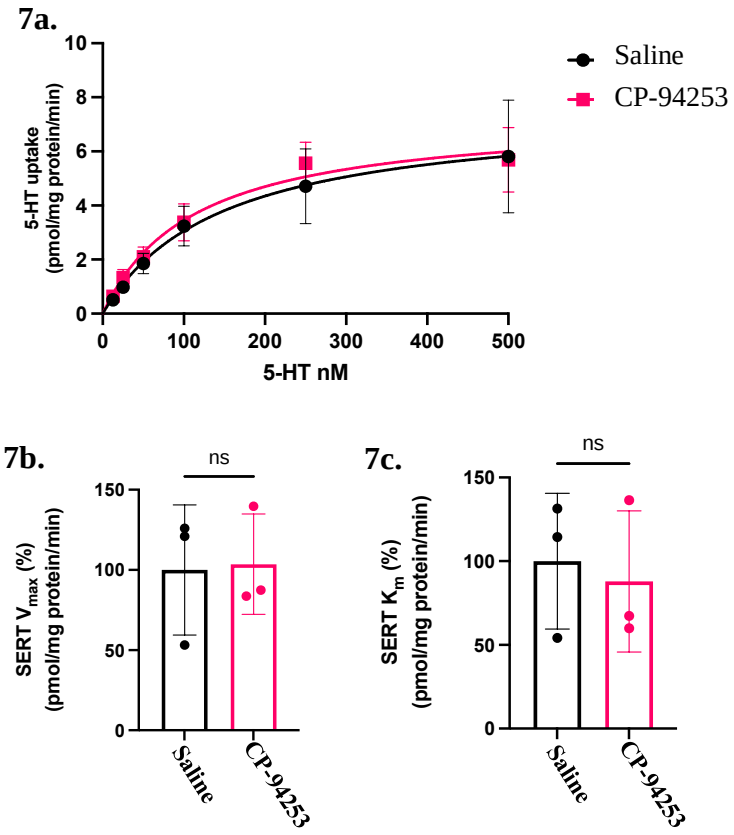


Figure 7a, b, c. Effects of CP-94253 on SERT Kinetics in Female mice (S44A/S48A Knock In Striatum N= 3). S44A/S48A Knock In female mice received injections of saline (shown in black) or CP-94253 at 1 mg/kg (shown in pink) 30 minutes before brain dissection. Serotonin (5-HT) kinetics assay was performed in triplicate, with data points representing the average for each concentration across all experiments. Non-linear curve fits of uptake data were generated for vehicle (N=3) and CP-94253-treated (N=3) groups. Statistical analysis was conducted using paired t-tests (*P < 0.05). Panel (a): Non-linear curve fits illustrating 5-HT uptake kinetics. Panel (b): Comparison of maximum uptake velocity (V_{max}) between vehicle and CP-94253-treated groups. Panel (c): Comparison of affinity (K_m) for the SERT under both conditions. ns - non-significant.

While, in wild type females, hippocampus treatment with CP-94253 resulted in a significant increase in the maximal velocity (V_{max}) (t=2.919, df=5; *P* =0.0433) and non-significant changes in K_m between vehicle and treatment (t=1.353, df=5; *P* =0.234).

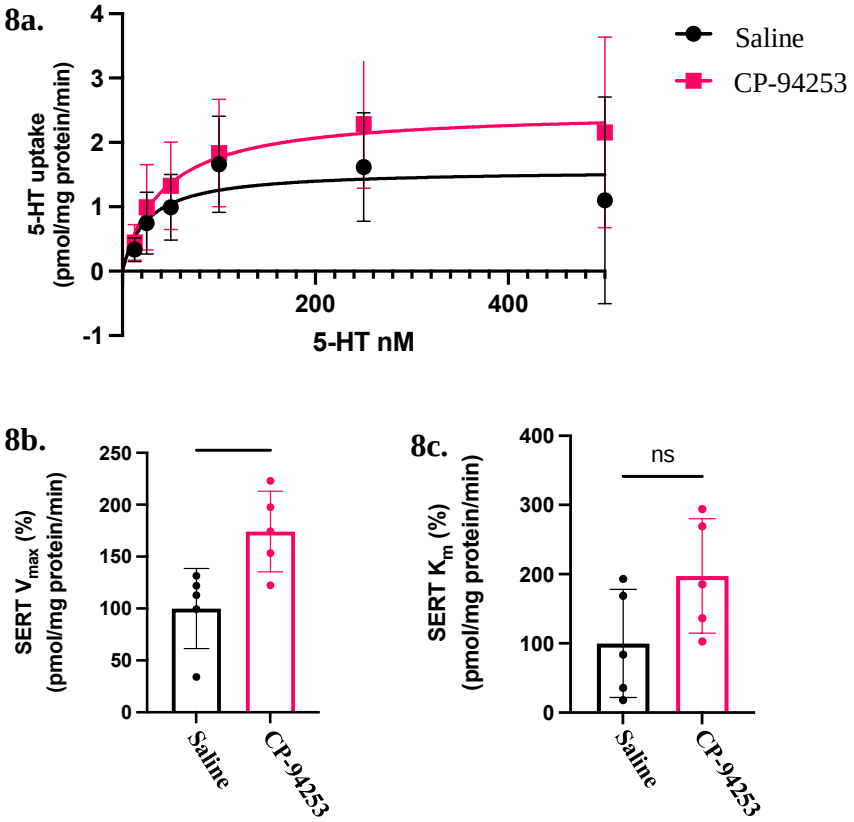


Figure 8a, b, c. Effects of CP-94253 on SERT Kinetics in Female mice (Wild Type Hippocampus N= 5). Wild Type Female mice received injections of saline (shown in black) or CP-94253 at 1 mg/kg (shown in pink) 30 minutes before brain dissection. Serotonin (5-HT) kinetics assay was performed in triplicate, with data points representing the average for each concentration across all experiments. Non-linear curve fits of uptake data were generated for vehicle (N=5) and CP-94253-treated (N=5) groups. Statistical analysis was conducted using paired t-tests (*P < 0.05). Panel (a): Non-linear curve fits illustrating 5-HT uptake kinetics. Panel (b): Comparison of V_{max} between vehicle and CP-94253-treated groups. Panel (c): Comparison K_m for the (SERT) under both conditions. *P = 0.0433 for V_{max} of CP-94253 treated mice. ns - non-significant.

In S44A/S48A Knock In females, hippocampus treatment with CP-94253 showed no difference between the maximal velocity (V_{max}) (t=1.739, df=2; *P* =0.2241) and non-significant changes in Km between vehicle and treatment (t=1.456, df=2; *P* =0.2826).

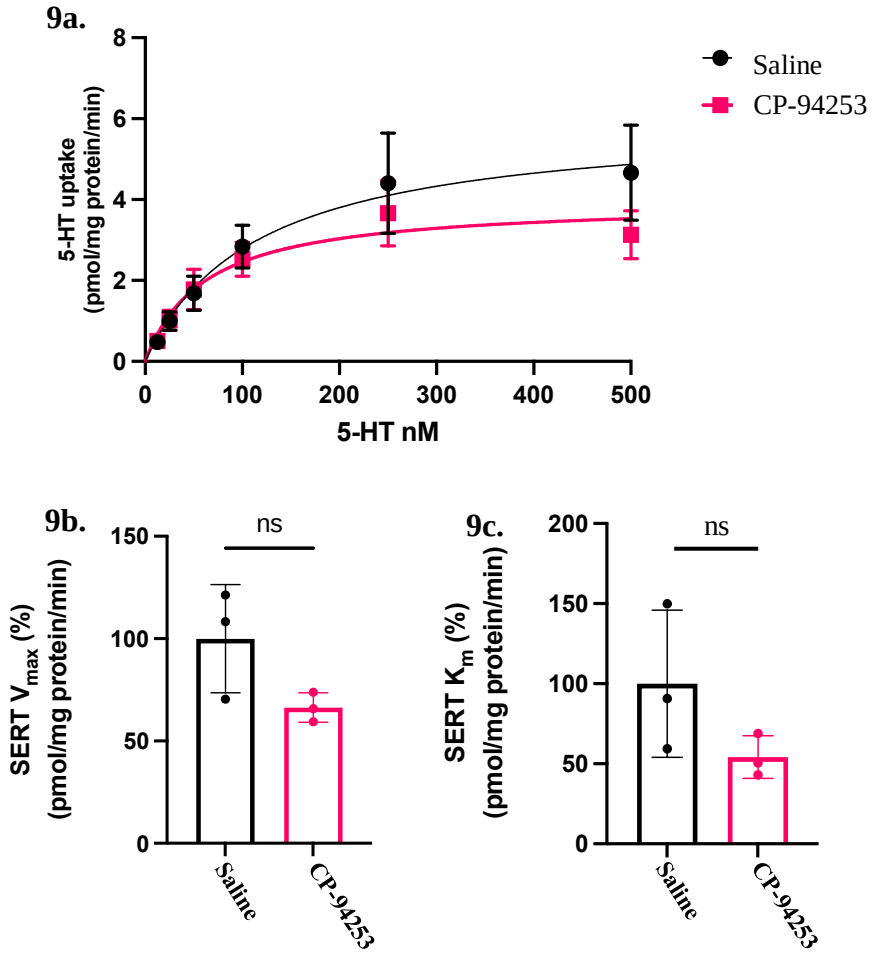


Figure 9a, b, c. Effects of CP-94253 on SERT Kinetics in Female mice (S44A/S48A Knock In Hippocampus N= 3). S44A/S48A Knock In Female mice received injections of saline (shown in black) or CP-94253 at 1 mg/kg (shown in pink) 30 minutes before brain dissection. Serotonin (5-HT) kinetics assay was performed in triplicate, with data points representing the average for each concentration across all experiments. Non-linear curve fits of uptake data were generated for vehicle (N=3) and CP-94253-treated (N=3) groups. Statistical analysis was conducted using paired t-tests (*P < 0.05). Panel (a): Non-linear curve fits illustrating 5-HT uptake kinetics. Panel (b): Comparison of V_{max} between vehicle and CP-94253-treated groups. Panel (c): Comparison of K_m for the SERT under both conditions. ns - non-significant.

DISCUSSION

The primary objective of this study was to investigate whether the GSK3β regulated phosphorylation sites S44/S48 play a role in 5-HT_{1B} autoreceptor mediated SERT regulation in female mice.

Sex differences in the 5-HT system have been previously documented in both rodents and humans, indicating the complexity of these interactions.[107] Not only 5-HT, but also gonadal hormones like estrogen and progesterone significantly impact mood modulation.[108,109]

Research shows that females are two times more prone to depression and anxiety disorders as compared to males, these conditions are often linked to abnormalities in the serotonin (5-HT) system.[110,111]

Additionally, CSF studies suggest that brain 5-HT metabolism rates are higher in females than in males as identified by increased 5-hydroxyindoleacetic acid (5-HIAA). Furthermore, differences in the behavioral outcomes have been observed using 5-HT_{1B} knock out females and males in the tail suspension test and force swim test, with females showing a more antidepressant like phenotype as compared to males, indicating a potential sex difference in the receptor activity.[107]

Although, the effect of activation of 5-HT_{1B} autoreceptor has been previously examined in males in our lab (unpublished data), given these differences my thesis

specifically targets female mice to better understand sex-specific mechanisms in 5-HT regulation involving the interplay between 5HT1B autoreceptors, SERT and GSK3β with a focus on the potential involvement of Serine44 and Serine48 phosphorylation sites on the transporter on activation of 5-HT_{1B} autoreceptor using CP-94253 (1mg/kg), a 5-HT_{1B} agonist. According to our findings, on activation of 5-HT_{1B} auto receptors in wild type females, there was seen an increase in SERT mediated uptake in both striatum and hippocampus, but these increases were not statistically significant. At the same time, no difference was seen in SERT S44A/S48A Knock In females between the vehicle and treated groups.

Although not statistically significant, the observed increase in SERT-mediated uptake in both the striatum and hippocampus following 5-HT_{1B} autoreceptor activation might be attributed to a weaker or more variable response due to hormone variance (cycle) in females. This trend suggests a regulatory mechanism consistent with the expected effect of 5-HT_{1B} autoreceptor activation on SERT function, as observed in males. The absence of a significant difference between the Knock In female control and treated groups indicates that the S44A/S48A mutations on the transporter may be crucial for 5-HT_{1B} autoreceptor-mediated SERT regulation. These results potentially highlight sex differences in this

regulatory pathway and underscore the need for further investigation with a larger sample size to fully elucidate these sex-specific mechanisms in 5-HT regulation. Next, kinetic studies revealed a significant increase in Vmax in the hippocampus and not striatum of wild-type females treated with CP-94253, without significant changes in Km. This indicates that 5-HT_{1B} autoreceptor activation may increase the number or efficiency of plasma membrane resident functional transporters in this region without altering their affinity for 5-HT. The lack of significant kinetic changes in the striatum indicates possible regional differences in this regulatory mechanism. This observed increase potentially might be due to differences in receptor densities or region-specific differences in intracellular signaling pathway. Importantly, SERT S44A/S48A Knock In females showed no significant differences in uptake or kinetics between vehicle and CP-94253 treated groups. This suggests that the Serine44 and Serine48 phosphorylation sites are crucial for 5-HT_{1B} autoreceptor-mediated regulation of SERT, potentially through GSK3β-mediated phosphorylation.

The less pronounced effects observed in females compared to previous male studies highlight the importance of considering sex as a biological variable in neuropharmacological research. These differences could be attributed to various factors, including hormonal influences, variations in receptor density, or alternative regulatory mechanisms in females. These results underscore the need for further

insights into the sex specific nature of 5-HT regulation in context of depression and anxiety like disorders.

Future directions for this study include examining the mechanisms underlying the increased SERT uptake observed, using biotinylation and Western blot studies to label membrane-bound proteins and detect changes in protein expression and localization following CP-94253 treatment. Previous research in our lab using male mice demonstrated that 5-HT_{1B} receptor activation leads to decreased SERT phosphorylation and increased SERT trafficking to the membrane, resulting in enhanced uptake. In SERT S44A/S48A mutants, increased uptake was observed even in control conditions, indicating the critical role of these sites in SERT regulation. Extending this investigation to female mice will provide insights into sex-specific mechanisms of 5-HT regulation, highlighting the importance of S44A/S48A sites and informing potential sex-specific therapeutic strategies.

In terms of behavior, $5HT_{1B}$ autoreceptor is implicated in a wide range of psychological effects including satiety, sleep, locomotor activity, body temperature regulation, memory modulation and aggression which are all also closely related to MDD. Apart from these, one of the important aspects of not just MDD but also commonly seen in ASD and PTSD is social isolation.[114–116] Studies suggest that social isolation behavioral deficits may be reversed using $5HT_{1B}$ agonists. [117] In

this context, social isolation can serve as a behavioral model to investigate whether S44/S48 sites on SERT mediate the effects of 5HT1B receptor activation on anxiety like behaviors observed in mice. If CP-94253 increases escape attempts and heightens anxiety-like behaviors in wildtype mice compared to vehicle controls, but this effect is absent or diminished in the SERT-A44/A48 knock-ins, it would suggest that the Ser44 and Ser48 phosphorylation sites on SERT mediate these behavioral consequences of 5-HT_{1B} activation in socially isolated mice. Additionally, the use of GSK3β inhibitor or GSKβ conditional knockout in serotonin neurons in mice could be used to investigate further if this effect is mediated through the GSK3 pathway. It could provide insights linking the $5HT_{1B}$ receptor activation and downstream signaling linked SERT regulation to stress induced behaviors. This behavioral data from the social isolation experiment would possibly allow us to functionally validate the significance of the SERT phosphorylation sites and their regulation by the 5-HT_{1B} receptor and GSK3 pathways in modulating serotonergic neurotransmission and mood-related behaviors.

CONCLUSION

In conclusion, this study provides important insights into the gender and region-specific mechanisms of 5-HT regulation in female mice. Despite not reaching statistical significance, the trend towards increased SERT-mediated uptake in the striatum and hippocampus following 5-HT1B autoreceptor activation suggests the presence of a complex regulatory mechanism that may be more variable in females. The lack of difference in SERT uptake and kinetics between vehicle and CP-94253 treated groups in SERT S44A/S48A Knock In females underscores the critical role of S44/S48 phosphorylation sites in 5-HT_{1B} autoreceptor-mediated SERT regulation, potentially through GSK3α/β-mediated pathways.

Additionally, the significant increase in Vmax in the hippocampus, but not in the striatum, indicates potential regional differences in this regulatory mechanism, which could be attributed to variations in receptor density or region-specific intracellular signaling pathways highlighting the complexity of 5-HT regulation, influenced by varied factors such as hormonal effects, receptor density, and alternative regulatory mechanisms in females.

Focusing on the 5-HT_{1B} receptor in relation to the GSK3β pathway, presents a promising avenue for treatments of mood disorders like depression and anxiety. Targeting the 5-HT_{1B} autoreceptor modulates 5-HT release and uptake, while

GSK3α/β inhibitors, which also play a role in SERT regulation, could lead to improved 5-HT signaling. Additionally, the S44/S48 phosphorylation sites on SERT also provide insights into how developing drugs that modulate protein kinases involved in SERT phosphorylation could provide new treatment options. These strategies highlight the importance of understanding sex differences in serotonergic function and pave the way for sex-specific therapeutic approaches.

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