The Identification and Characterization of Serotonin Receptors in Cancer and Their Response to Serotonin and Selective Serotonin Reuptake Inhibitors

by

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Submitted in Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

in the

Health Sciences

Program

YOUNGSTOWN STATE UNIVERSITY

August 2024

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ABSTRACT

This study investigates the expression profiles of serotonin receptors and the serotonin transporter (SLC6A4) across various cancer cell lines (A549, Hs 578T, MDA-MB-231, MCF7, and SK-N-AS) and examines the modulatory effects of serotonin (5HT) and selective serotonin reuptake inhibitors (SSRIs) on these profiles. Utilizing immunofluorescence imaging and flow cytometry, we determined the baseline expression levels of serotonin receptors ($5HTR_{1B}$, $5HTR_{1D}$, $5HTR_{1E}$, $5HTR_{2A}$, $5HTR_{2B}$, $5HTR_{2C}$, $5HTR_{5A}$, and $5HTR_{7}$) and the serotonin transporter (SLC6A4). Our results revealed that serotonin treatment induces a transient upregulation of receptor expression, with a peak at 24 hours and a return to baseline by 48 hours. This suggests a temporary enhancement of serotonergic signaling in response to increased extracellular serotonin levels. In contrast, SSRI treatment led to a sustained decrease in receptor expression, particularly notable in A549, Hs 578T, and MCF7 cell lines, indicating potential receptor desensitization and downregulation. The MDA-MB-231 cell line exhibited less prominent inhibition, suggesting possible resistance mechanisms. This comprehensive analysis highlights the complex regulatory mechanisms governing serotonin receptor expression and underscores the potential of SSRIs as adjunctive anticancer therapies. By establishing baseline expression levels and documenting the transient and long-term effects of serotonin and SSRIs on these receptors, this study lays the foundation for future research aimed at understanding the role of serotonin signaling in cancer progression and developing novel therapeutic strategies targeting serotonin pathways.

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Abbreviations

5' Adenosine Monophosphate-Activated Protein Kinase (AMPK)
5-hydroxytryptophan (5-HTP)
Activating transcription factor 4 (ATF4)
Aldehyde Dehydrogenase And Monoamine Oxidase-A (ALDH-2)
Aromatic Amino Acid Decarboxylase (AADC)
Cerebrospinal Fluid (CSF)
Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition, Text Revision (DSM-5-TR)
Hypoxia-Inducible Factor 1-alpha (HIF1α)
Inositol Trisphosphate (IP3)
International Classification of Diseases 11th Revision (ICD-11)
Kappa Light Polypeptide Gene Enhancer In B-Cells Inhibitor Beta (IκB-β),
Major Depressive Disorder (MDD)
Matrix Metallopeptidase-9 (MMP9)
Matrix Metalloproteinase 12 (MMP12)
Mitogen-Activated Protein Kinase (MAPK)
Reactive Oxygen Species (ros)
Monoamine Oxidase A (MAO-A)
Monoamine Oxidase Inhibitors (MAOIs)
Myelocytomatosis Oncogene (c-MYC)
Nuclear Factor-κB (NF-κB)
Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells Inhibitor Alpha (IκB-α)
Obsessive–Compulsive Disorder (OCD)
Phosphoinositide 3-Kinases (PI3Ks)
Protein Kinase B (Akt)
Protein Kinase C (PKC)
Serotonin (5-hydroxytryptamine, 5HT)
Serotonin Receptors (5HTRs)
Serotonin Transporter (SLC6A4)
Serotonin/Noradrenaline Reuptake Inhibitors (SNRIs)
Tricyclic Antidepressants (TCAs)
Tryptophan Hydroxylase (TPH)
Urokinase-Type Plasminogen Activator (uPA)
Vascular Endothelial Growth Factor (VEGF)
Vesicular Monoamine Transporter Isoform 2 (VMAT2)
World Mental Health (WMH)

PREFACE

I would like to acknowledge the people who have believed in me and guided me throughout my career. Dr. Steelant, thank you for taking a chance with me all those years ago, when I was a failing undergrad, and struggling to my place. Thank you for always putting your neck out for me and continuing to support me through my master's and PhD. Dr. Sev, you have always pushed me to be the greatest I could be and always picked me up when I was down. Thank you for helping me become proud of myself, my work and my research. More importantly, thank you for showing me the value of going the extra mile and the folly of my occasional… Lapses of laziness. I hope to continue to make you both proud. Dr. Ken Learman, I cannot thank you enough for your patience and support throughout the years that you have shown me as I transitioned into the program and a different field. I hope to keep making you all proud and to continue forward with all the knowledge and skills that you have each given me.

I also want to thank my colleagues and best friends, Alexander D. Huber and Zachary Marinelli. You guys have stuck through my rantings and raving about my work this whole time, and your inputs has always been helpful. And to Amanda Hall, thank you for being at my side from the start and being by biggest supporter.

And finally, to my mother and the strongest person I know, Terry Oliva, for being my inspiration for this project and above all for helping me become the man I am today.

1 Introduction

The term depression is often used to refer to one of the many types of depressive disorders described in the *Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition, Text Revision (DSM-5-TR)* and in the *International Classification of Diseases 11th Revision (ICD-11)*. Depression encompasses about 13% of all mental illnesses and accounts for over half of all outpatients' psychological visits.[1] Depressive disorders are generally characterized by severe and persistent sadness which interferes with one's function and is associated with a decreased interest and related pleasures of usual activities. While the exact mechanisms are not fully elucidated, alterations in normal neuroendocrine and neurotransmitter functions are believed to lead to the development of depressed phenotypes.[2,3] Among these proposed mechanisms is the dysregulation of serotonin and its associated receptors leading to the development of depression. Serotonin (5-hydroxytryptamine, 5HT) is a monoamine produced from tryptophan, an essential amino acid.[4] Serotonin is mainly located in the periphery with less than 1% of total serotonin found in circulation.[5,6] Depression has also been linked to several comorbidities, specifically a positive association with cancer development, treatment, recovery, and remission.[7–9] Moreover, several studies have identified that serotonin as well as certain serotonin receptors (5HTRs) play a role in cancer development and progression.[10,11]

The development of depression in patients with cancer has historically been under diagnosed, under treated, and partially ignored, due to the fact that depression was seen as the appropriate reaction to such a diagnosis.[3,12] Similarly, many of the neurovegetative as well as the cognitive emotional symptoms seen in depression are often attributed to cancer itself. The prevalence of depression in cancer patients directly correlates to the severity of

the disease.[8,13] Moreover, many of the associated symptoms of cancer such as chronic pain and fatigue only exacerbate the issue. However, the results from the literature are clear, there is strong correlation between the extent of a patient's depression to their prognosis, receptiveness to treatment and the probability of reoccurrence. However, what is not fully understood are the biochemical mechanisms of depression and their effect on cancer development and progression. Specifically, the way that 5HT and its associated receptors as a whole affect cancer progression and development is not clearly understood. Therefore, the purpose of this study is to determine the effects of 5HT and depression on cancer progression. Specifically, the categorization of the different serotonin receptors expressed in different types of cancer and the combined effects on cancer development and progression.

1.1 Depression

1.1.1 Characterization of depression

Major Depressive Disorder (MDD) is the most common mental disorder and affects about 200 million people a year in the United States.[14] Patients with MDD generally present with depressed mood, anhedonia, issues with sleep, and recurrent suicidal ideations. People with depression experience significant psychological and physical comorbidities such as anxiety, cardiovascular disease, obesity, and cancer.[15–18] Additionally, mental disorders and especially depression have historically been a source of shame and stigma for many suffering from the disease.[19] This, combined with the genetic, environmental, psychological and physiological factors that are associated with depression make it very difficult to diagnose, let alone provide a definitive mechanism for its development.[20,21]

The *World Mental Health Survey (WMH)*, which utilized retrospective evaluations on a global scale, indicated a lifetime prevalence of around 10%.[22] However, retrospective studies are prone to under-estimations due to under-reporting and recall biases. One prospective of about 32 years suggested a prevalence of depression of ≥30%, whereas other multinational systematic reviews focusing on shorter timelines estimated the prevalence of depression to be about 5% globally.[23,24]

As the estimated prevalence of depression varies with study design, so do the rates of its development in different groups. Several studies have shown that the prevalence of depression also fluctuates with socio-economic status, race, and gender.[25,26] This is especially true for gender, where depression is almost twice as high in women than in men, regardless of race, country, and socio-economic status.[27] Additionally, children of individuals with depression are at least 30% more likely to develop depression by early adulthood, which is almost twice the risk of people who do not have parents with a history of depression.[21,28,29]

Usually, depression presents in adolescence and early adulthood with depressive episodes lasting between 3-6 months, of which, 60% resolve within a year.[30] It should be noted that longer depressive episodes are associated with the length of time between the development of symptoms and the receiving of care as well as the severity of the depressive episodes. This may contribute to the fact that depression is correlated with premature mortality, primarily attributed to elevated rates of comorbid conditions in contrast to the general population.[23,31,32] People with depression are also at higher risk of suicidal ideations, planning, and attempts of suicide.[33] Indeed, the risk of suicide due to depression is 20 times more likely as compared to those without.[34]

The key method for diagnosing MDD, involves applying the operational diagnostic criteria outlined in the DSM-5-TR and the ICD-11.[14] The DSM-5-TR serves its main purpose within the realm of scientific research, aiming to provide a foundation for studies. On the other hand, the ICD focuses on refining the practical usefulness of diverse diagnoses, catering to the clinical intricacies of real-world practice.

Individuals suspected of having depression undergo an assessment to ascertain the presence of MDD symptoms over a span of two weeks. It is imperative during this evaluation to rule out symptoms stemming from medication side effects or other psychiatric/medical conditions. Additionally, the severity of symptoms should surpass typical feelings of sadness. It is noteworthy that symptoms associated with grief and bereavement necessitate special attention. While the ICD-11 criteria allow for the diagnosis of depression in bereaved individuals, a higher threshold is set. The DSM-5-TR introduced a new category named 'prolonged grief disorder', characterized by significant distress or impairment persisting for over 12 months following the loss of a close relation. While distinguishing between MDD and prolonged grief disorder is crucial, both can be diagnosed using DSM-5-TR criteria if the requirements for both disorders are met. Due to the diverse nature of depression, there have been continuous efforts to delineate subtypes. The DSM-5-TR accomplishes this through specifiers.

To meet the diagnostic criteria for MDD according to the DSM-5-TR, an individual must exhibit at least five of the following symptoms consistently over a two-week period.[213] These symptoms must occur nearly every day and signify a departure from previous functioning. Additionally, they should lead to significant distress or impairment, not be attributed to substance use or another medical condition, and not be more

appropriately explained by schizophrenia spectrum disorders. Moreover, there should be no history of a manic or hypomanic episode.

- *"Depressed mood most of the day, nearly every day, as indicated by either subjective report or observations made by others*
- *Markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day*
- *Significant weight loss when not dieting or weight gain (e.g., a change of more than 5% of body weight in a month) or decrease or increase in appetite nearly every day*
- *Insomnia or hypersomnia nearly every day*
- *Psychomotor agitation or delay nearly every day*
- *Fatigue or loss of energy nearly every day*
- *Feelings of worthlessness or excessive or inappropriate guilt nearly every day*
- *Diminished ability to think or concentrate, or indecisiveness, nearly every day*
- *Recurrent thoughts of death (not just fear of dying), recurrent suicidal ideation without a specific plan, or a suicide attempt or a specific plan for committing suicide"*

To meet the diagnostic criteria for MDD according to the ICD-11 an individual must display a minimum of five symptoms, including one from the affective cluster.[214] These symptoms must persist for most of the day, almost every day, over a span of at least two weeks. They should also lead to significant impairment and not be primarily attributable to

bereavement, another medical condition, or substance or medication use. Additionally, the individual should not meet the criteria for a mixed episode.

- *"Affective Cluster:*
    - *Depressed mood*
    - *Markedly diminished interest or pleasure in activities*
- *Cognitive Behavioral Cluster:*
    - *Reduced ability to concentrate and sustain attention to tasks, or marked indecisiveness*
    - *Feelings of low self-worth or excessive and inappropriate guilt*
    - *Hopelessness*
    - *Recurrent thoughts of death, recurrent suicidal ideation, or evidence of attempted suicide*
- *Neurovegetative Cluster:*
    - *Significantly disrupted sleep or excessive sleep*
    - *Significant change in appetite or significant weight change*
    - *Psychomotor agitation or hindrance*
    - *Reduced energy, fatigue or marked tiredness"*

Regardless of diagnostic criteria, it is critical to understand that depressive symptoms exist on a continuum and can vary depending on culture and social norms. This resulting variability and heterogeneity has led to the classification of depression into subtypes using specifiers. This is further complicated by subthreshold depressive states,

but still result in clinical dysfunction. Nevertheless, while often thought of as a singular event in a patient's life, depression is usually a lifetime spanning illness.

Individuals struggling with depression often struggle with a diminished quality of life, influenced not only by the disorder itself but also by associated medical comorbidities, socioeconomic factors, and impaired functional outcomes. The intricate nature of depression defies a singular explanation through any established biological or environmental pathway.[35–37] Yet biochemically, the discourse on mechanisms in depression, has largely revolved around the monoamine hypothesis. This theory suggests that deficiencies in monoaminergic neurotransmitters, such as serotonin, noradrenaline, and dopamine, play a role, at least in part, in the development of depression.[38]

1.1.2 Serotonin and its role in depression

Serotonin is a monoamine synthesized from the essential amino acid tryptophan, which is actively co-transported with other neutral large amino acids from the blood to the brain.[39] Tryptophan is hydrolyzed into 5-hydroxytryptophan (5-HTP) by the rate-limiting enzyme tryptophan hydroxylase (TPH). It is then decarboxylated to serotonin by aromatic amino acid decarboxylase (Figure 1). Interestingly, there are two different tryptophan hydroxylase enzymes, TPH1 and TPH2, which serve as the basis for two different populations of serotonin in the body.[40,41] With TPH1 being responsible for the synthesis of serotonin in the periphery by the enterochromaffin cells of the gut and pineal gland, and TPH2 is synthesized in the serotonergic neurons of raphe nuclei of the brainstem and the myenteric neurons in the gastrointestinal tract.[40–42] Due to this fact, the two serotonergic systems are independent of each other in function and regulation.

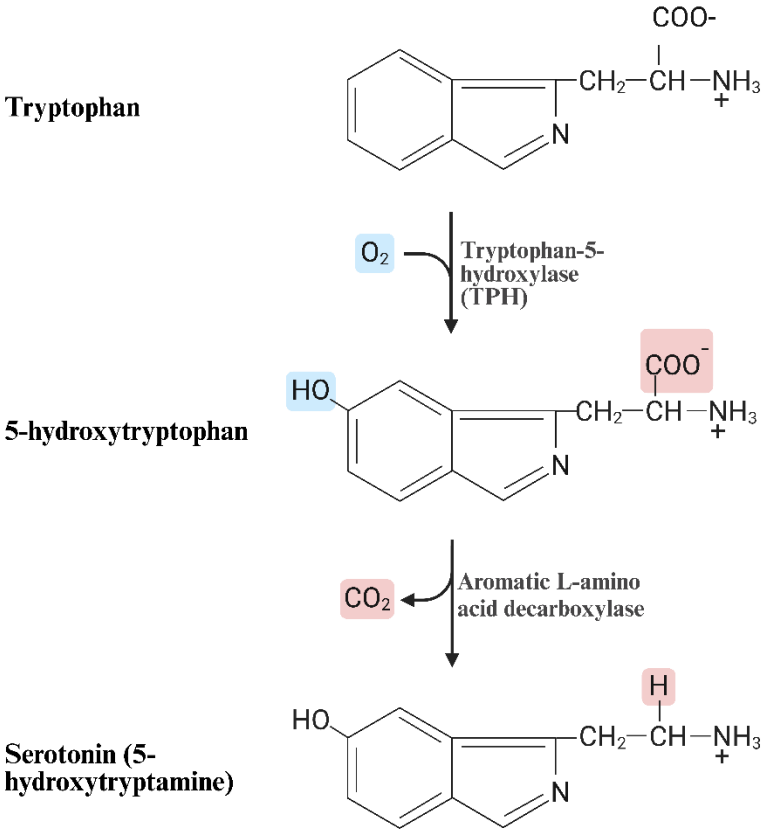


Figure 1 Serotonin Synthesis

Tryptophan is transported into the cytoplasm of serotonergic neurons or enterochromaffin cells, where it is converted to 5-hydroxytryptophan (5-HTP) by the enzyme tryptophan hydroxylase (TPH). 5-HTP is then decarboxylated by aromatic L-amino acid decarboxylase (AADC) to form serotonin.

Most serotonin synthesis (>90%) occurs in the peripheral enterochromaffin cells of the gastrointestinal tract with only a fraction of total serotonin (<1%) circulating freely in the blood.[43] In the digestive system, serotonin acts as a hormone and stimulates gastric emptying, intestinal secretions, and improving gut motility.[5,44] In the cardiovascular system, serotonin increases intracellular calcium in cardiac myocytes triggering tachyarrhythmias and also contributes to vasodilation. Additionally, serotonin is stored in platelets where it is vital for cellular repair as well as being a known mitogenic factor for multiple cancer and normal cell types.[45,46]

In the central nervous system, serotonergic neurons are located in the midbrain, specifically within the raphe nucleus of the brain stem, with axons extending to various brain structures, predominantly in the cortex, limbic system, basal ganglia, and spinal cord.[47,48] Following its synthesis in these serotonergic neurons, 5HT is taken up into vesicles in the axon terminal by vesicular monoamine transporter isoform 2 (VMAT2). Then the stimulation of an action potential results in the calcium-dependent exocytotic release of serotonin from presynaptic vesicles into the synaptic cleft, where serotonin interacts with both postsynaptic serotonergic receptors and presynaptic autoreceptors (Figure 2).[49] The termination of the action of serotonin is facilitated by the serotonin transporter (SLC6A4) through the co-transport of sodium and chloride with serotonin which is then exchanged with intracellular potassium. Finally, after reuptake, 5HT can be recycled by aldehyde dehydrogenase and monoamine oxidase-A (ALDH-2).

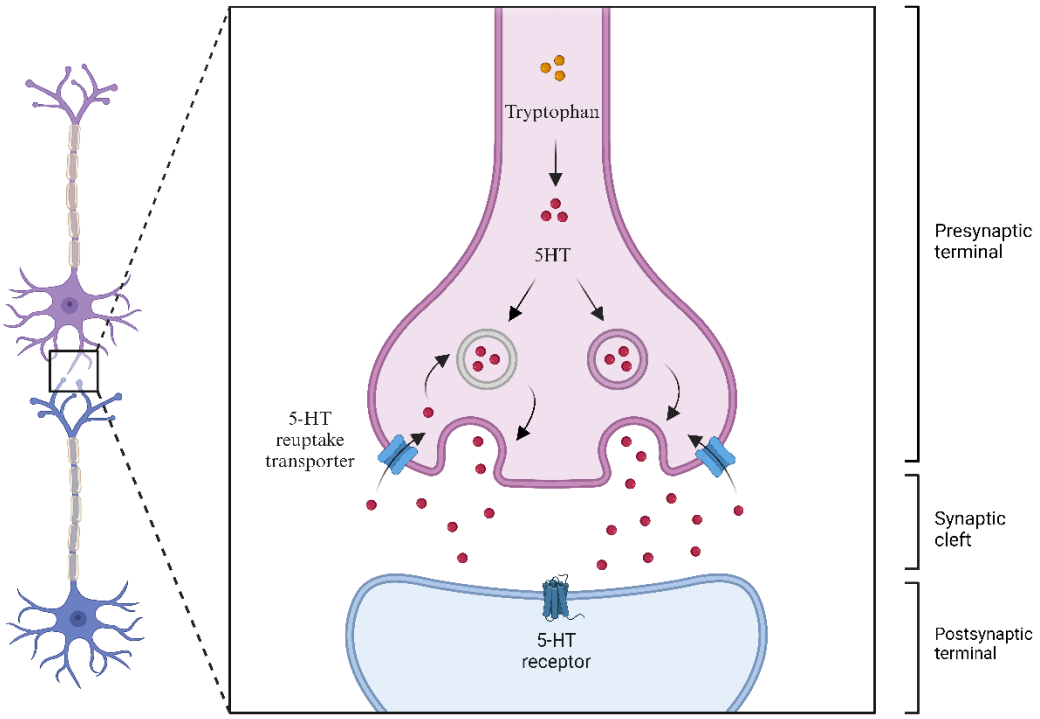


Figure 2 Serotonin Signaling in the Central Nervous System

Following its synthesis, serotonin is released from presynaptic neurons and binds to various serotonin receptor subtypes located on the postsynaptic membrane. Receptor activation triggers distinct intracellular signaling cascades, mediating diverse physiological functions.

For over fifty years, the serotonin theory of depression has been the source of significant discourse.[35] The initial investigations into the pathology of depression centered on brain neurochemistry. This theory posits that an imbalance of brain serotonin levels leads to the development of depression. In the later 1960s the broader monoamine hypothesis stated that in the synaptic cleft, the concentration of monoamines such as serotonin and noradrenaline are decreased in a depressive state.[50,51] This monoamine hypothesis of depression, stemmed from several pharmacokinetic clinical observations. Reserpine, an antihypertensive medication used to manage high blood pressure, also had

the side effect of depleting monoamines such as serotonin in the brain which induced depressive episodes in patients who had no previous history of depression.[50] Further credence was lent to the monoamine hypothesis with the discovery of the first-generation antidepressants: tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs). These medications were shown to elicit a short-term rise in the concentration of monoamines, specifically serotonin in the synaptic cleft.[51] Further supporting this hypothesis are subsequent studies revealing a decrease in serotonin metabolite concentrations within the cerebrospinal fluid of depressed patients.[52]

Despite the initial enthusiasm surrounding the monoamine hypothesis, studies investigating deficiencies in serotonin did not yield definitive results. Serotonin and serotonin metabolites in plasma, urine, and cerebrospinal fluid at the time, did not consistently show significant reductions in depressed individuals, nor did post-mortem studies on the brains of depressed patients provide conclusive evidence for a straightforward deficiency.[52,53]

One recent development that challenges the simplicity of the monoamine hypothesis is the discovery of the neuronal-specific isoform of the enzyme tryptophan hydroxylase, TPH2.[54] Previously, research focused on the brain-specific TPH1, thought to be the primary regulator of serotonin synthesis. However, the identification of TPH2, with its broader presence in peripheral neurons, suggests a more complex role for serotonin in mood regulation beyond just brain levels, and may suggest why prior studies focusing on total enzyme activity and total serotonin concentrations did not show differences between patients with and without depression. This is further supported by a Positron Emission Tomographic study which used harmine-labeled with carbon-11, a radioligand selective

for Monoamine oxidase A (MAO-A), an enzyme that metabolizes monoamines, such as serotonin.[55] This study found that in 17 depressed and 17 healthy patients, MAO-A was significantly elevated in every brain region assessed (prefrontal cortex, anterior cingulate cortex, posterior cingulate cortex, caudate, putamen, thalamus, anterior temporal cortex, midbrain, hippocampus) by an average of 34%.

The significance of tryptophan availability for brain serotonin synthesis has fueled attempts to manipulate serotonin levels acutely through diet. One common technique is "tryptophan depletion," which involves administering a large dose of amino acids that lack tryptophan. This approach significantly reduces blood tryptophan levels by 80% within 8-12 hours.[56] Studies in both animals and humans demonstrate a significant decrease in brain serotonin synthesis following tryptophan depletion.[57,58]

The role of serotonin in the development of depression has been further examined through tryptophan depletion studies. Notably, research shows patients without depression risk factors will not experience significant mood decline after tryptophan depletion.[59] This suggests that simply lowering brain serotonin is not sufficient to induce depression. However, this is complicated when tryptophan depletion can trigger depressive episodes in individuals recovering from depression who are medication and therapy-free for extended periods. This suggests that while a decrease in brain serotonin might not be necessary or sufficient for initial depression onset, it does contribute to the maintenance of a depressive state and relapse in previously depressed patients. It is also possible that reduced serotonin activity interacts with pre-existing neurobiological vulnerabilities, potentially affecting the regulation of key neural networks resulting in depression.[60] Overall, these conclusions are further supported by the delayed onset of action for

antidepressant treatments. While these medications can elevate monoamine levels within minutes, their therapeutic effects typically take weeks to manifest. This time lag suggests that the mechanism by which antidepressants work goes beyond simply increasing serotonin.

While the serotonin hypothesis has significantly influenced our understanding of depression, it is essential to recognize the multifaceted nature of the depression. Depression is shaped by a complex interplay of biological, psychological, and environmental factors, and the serotonin hypothesis, while informative, does not offer a comprehensive explanation for all facets of depression. Even so, it remains a valuable framework guiding both research endeavors and therapeutic interventions aimed at addressing serotonin imbalances in individuals with depressive disorders. Indeed, investigations into serotonin receptors have underscored their role in modulating mood and emotional states.[61–63]

1.1.3 The serotonin transporter and receptors

As previously described, the serotonin hypothesis of depression has been a driving force in understanding the development of depression. At its core, this theory revolves around the notion that serotonin dysregulation is the main driver for the development of depression. The most robust body of evidence for this theory is the effectiveness of tricyclic antidepressants, monoamine oxidase inhibitors, and more commonly selective serotonin reuptake inhibitors in the treatment of depression.[64,65] It is believed that these drugs inhibit the action of the serotonin transporter and result in an increase of extracellular serotonin, suggesting that increasing synaptic serotonin is an effective form of depression treatment. As a result, the serotonin transporter (SLC6A4), and the 6 types of serotonin receptors (5HTRs) have been associated with the modulation of depression in humans. Six of the

serotonin receptor classes function as G-protein-coupled receptors, apart from the 5HTR3 receptor, which is distinct for being a ligand-gated sodium-potassium ion channel.[66] The specific function of each receptor type is influenced by its unique characteristics and the associated signaling pathway, of which 5HTR1,2,5,7 and the serotonin transporter are of particular interest to this field (Figure 3).

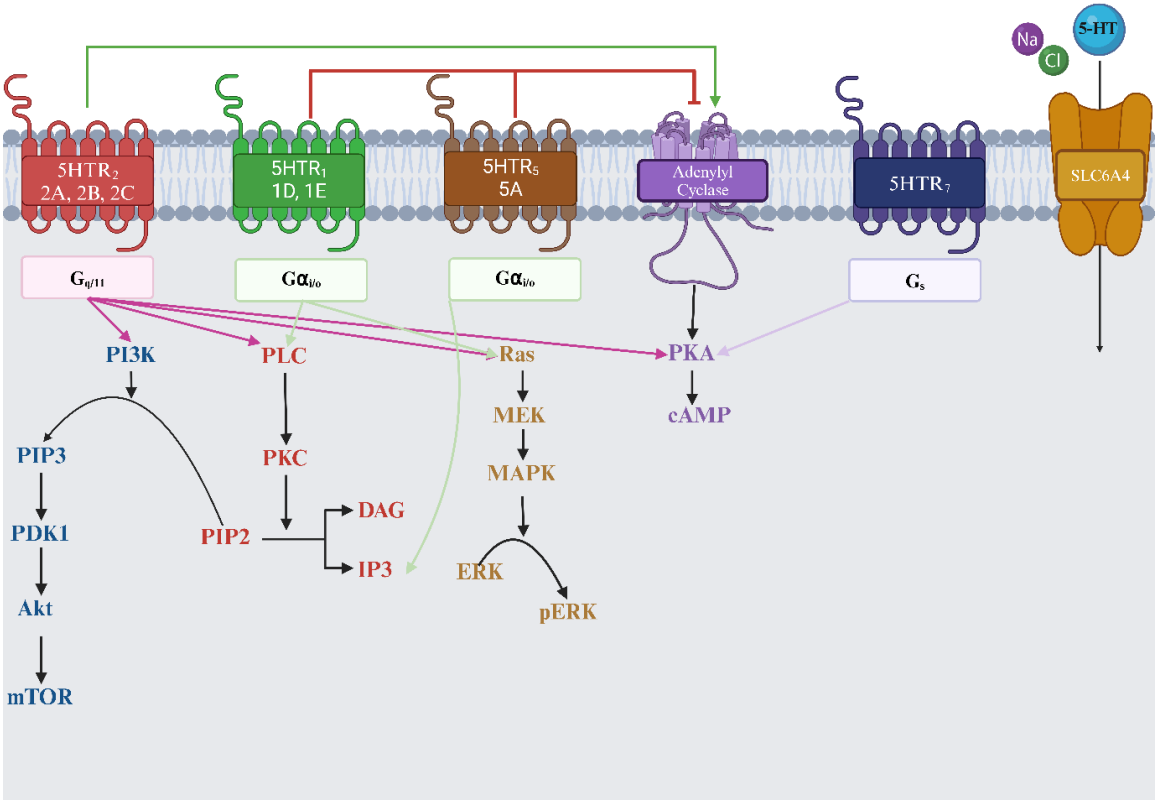


Figure 3 The Serotonin Transporter and Receptors.

This figure illustrates the interplay between the serotonin transporter and serotonin receptors in regulating neurotransmission. The serotonin transporter (SLC6A4), expressed on the presynaptic membrane, reabsorbs serotonin from the synaptic cleft, limiting its interaction with postsynaptic receptors. Different serotonin receptor subtypes, located on the postsynaptic membrane, initiate diverse cellular responses upon serotonin binding. This dynamic interplay between reuptake and receptor activation determines the overall strength and duration of serotonergic signaling.

The $5HTR_1$ receptor subfamily is the largest and encompasses the $5HTR_{1A}$, $5HTR_{1B}$, $5HTR_{1D}$, $5HTR_{1E}$ and $5HTR_{1F}$ receptor subtypes. These receptors share a high sequence homology (45-60%) and also couple with G_i/G_o to inhibit adenylyl cyclase activity and are primarily inhibitory in nature.[67,6861] Due to their widespread distribution and diverse signaling pathways, $5HTR_1$ receptors play a critical role in numerous physiological processes, including mood regulation, anxiety and pain perception.[69,70] Of the $5HTR_1$ receptors observed in this study, $5HTR_{1E}$ is perhaps the least understood and studied, but has been shown to be involved in cell survival and proliferation in multiple cancer types.[71–74] On the other hand, $5HTR_{1D}$ seems to be involved in neurotransmitter regulation and vasoconstriction in the periphery and cancer development.[45,61,72]

The $5HT_2$ receptor family is comprised of three subtypes: $5HTR_{2A}$, $5HTR_{2B}$, and $5HTR_{2C}$. Similar to the $5HTR_1$ receptor subfamily, $5HTR_2$ are found throughout the brain and in peripheral tissues. $5HTR_{2A}$ with its pronounced expression in the brain, especially in regions such as the cortex and hippocampus, is implicated in modulating mood, cognitive functions, and perception. $5HTR_{2A}$ receptors, both postsynaptic and recently discovered presynaptic heteroreceptors, couple via $G_q/11$ to the Inositol Trisphosphate (IP3)/ Protein Kinase C (PKC)/Calcium pathways, potentially influencing both serotonergic and glutamatergic neurotransmission.[75] Its activation by certain ligands, including hallucinogens, highlights its potential relevance in psychiatric and neurological disorders.[76–78] More importantly however, is the role that $5HTR_{2A}$ plays in the development of depression, especially since $5HTR_{2A}$ agonists and antagonists exhibit antidepressant like effects.[79] Conversely, $5HTR_{2B}$, which is mainly found in peripheral tissues such as the heart, stomach, and lungs, plays critical roles in embryonic development, cardiac

functionality, and gastrointestinal mobility.[80–82] $5HTR_{2C}$ is mainly involved in mood regulation, appetite, and circadian regulation. It is worth noting that it has been shown that an overactivity of $5HT_{2C}$ receptors may contribute to depressive and anxiety symptoms in some patients. Some of the initial anxiety caused by SSRIs is due to excessive signaling of $5HTR_{2C}$.[82–84] Additionally, stimulation of $5HTR_{2C}$ by serotonin may be responsible for many of the adverse side effects antidepressant medication, such as sertraline, paroxetine, venlafaxine, etc.[85]

The $5HTR_5$ receptor subfamily consists of two receptors, $5HTR_{5A}$ and $5HTR_{5B}$ and are also coupled with G_i/G_o to inhibit adenylyl cyclase activity. $5HTR_{5A}$ receptors are predominantly found in brain regions associated with memory and learning, influencing neurotransmitter release and synaptic plasticity.[86–88] However, the $5HTR_5$ receptor subfamily is perhaps the least studied and least well understood group, with $5HTR_{5B}$ not being found in humans and $5HTR_{5A}$ not being well studied.[89]

The serotonin transporter, a monoamine transporter protein, modulates serotonergic neurotransmission by removing 5HT from the extracellular space.[90,91] This is performed through the co-transport of sodium and chloride with serotonin which is then exchanged with intracellular potassium. While this serotonin reuptake is associated with neurons, it has also been identified in multiple other cell types, and especially in cancer cells.[11,46,90]

1.1.4 Selective serotonin receptor inhibitors

Antidepressants can be categorized into five primary types: tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs), serotonin/noradrenaline reuptake inhibitors (SNRIs), tetracyclic antidepressants, and selective serotonin reuptake inhibitors (SSRI) which are the most prescribed. SSRIs are usually prescribed as the first-

line drug treatment for depression due to their safety in case of overdose, overall tolerability, cost-effectiveness since generic alternatives are available, their versatile range of applications and have fewer side effects than MAOIs or TCAs. Additionally, SSRIs offer a therapeutic profile that extends beyond their traditional antidepressant effects. They have demonstrated effectiveness in treating conditions such as panic disorder, generalized anxiety disorders, obsessive–compulsive disorder (OCD), and bulimia. Moreover, promising results have been observed in addressing social phobia, post-traumatic stress disorder, premenstrual dysphoric disorder, migraine, and even dysthymia.[92–95] Most SSRIs share similar half-lives of around 24 hours, except for fluoxetine which is around 48 hours.[96,97] Additionally, the secondary metabolite of fluoxetine, norfluoxetine, has a half-life of 7-15 days in adults. SSRIs also all share a similar mechanism of action, selectively blocking the serotonin reuptake pumps, which leads to an increase in the availability of serotonin in the synaptic cleft and therefore an increase in serotonin receptor activity (Figure 4).

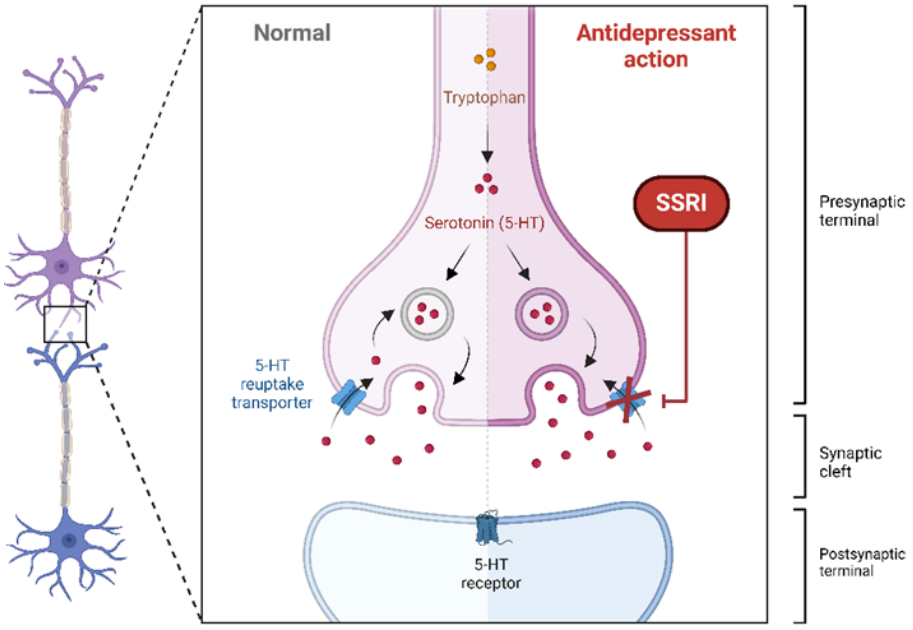


Figure 4 Selective Serotonin Reuptake Inhibitors and Serotonin Signaling

SSRIs bind competitively to the serotonin transporter (SLC6A4) located on the presynaptic membrane. By blocking SLC6A4, SSRIs prevent the reuptake of serotonin from the synaptic cleft, resulting in elevated serotonin levels. This increased availability of serotonin allows for more robust activation of postsynaptic serotonin receptors, potentially leading to therapeutic effects in mood disorders.

The primary mechanism of action attributed to SSRIs is generally believed to be their selective inhibition of the serotonin transporter, leading to an increase of serotonin in the synaptic cleft and an increase in synaptic activity, therefore prolonging the time that serotonin activates post-synaptic 5HT receptors.[96,98,99] Although SSRIs rapidly inhibit the reuptake of serotonin, their therapeutic effects in alleviating depression manifest with a delay of 2-4 weeks. The delayed onset of antidepressant action remains a topic of investigation, with a prevailing hypothesis attributing it to modulatory effects at the synapse. These modulations are thought to lead to the desensitization of $5HTR_{1A}$ serotonergic receptors, a receptor subtype strongly implicated in the pathophysiology of depression.[100–102] These studies have shown that, SSRIs lead to a delayed disinhibition of serotonergic neurotransmission in at least four key pathways that occur following

desensitization of $5HT_{1A}$ and $5HT_{1B}$ autoreceptors. Adding to this theory, a meta-analysis of *in vivo* microdialysis studies in rats examined the effects of SSRI administration on extracellular 5HT levels across various brain regions.[103] The findings revealed a region-specific response, with increases in 5HT observed in the prefrontal cortex and hippocampus within the first three days of treatment, while the frontal cortex exhibited a decrease. Overall, if SSRIs are administered over an extended period, the persistent elevated levels of serotonin in the dendritic region of serotonergic neurons leads to a desensitization of dendritic $5HT_{1A}$ receptors. Once these receptors become desensitized, serotonin can no longer readily inhibit neuronal impulse flow. This results in an increased release of serotonin from the terminal presynaptic region. However, this increase is delayed compared to the rise of serotonin in the dendritic region, as it takes time for dendritic serotonin to desensitize the $5HT_{1A}$ receptors. This delay may explain why SSRIs do not provide immediate relief for depression and anxiety. Regardless of their precise mechanism of action, the ability of SSRIS to increase serotonin levels and therefore increase the activity of serotonergic receptors give credence to their effectiveness.

Generally, citalopram, fluoxetine, and sertraline, are some of the most prescribed SSRIs.[104] Despite their shared mechanism of inhibiting serotonin reuptake, individual SSRIs exhibit distinct pharmacokinetic and pharmacodynamic profiles. These variations translate into unique side-effect characteristics and efficacy spectrums, rendering each SSRI suitable for specific clinical presentations. Selection of the optimal SSRI necessitates a patient-centered approach, carefully considering both the core clinical picture and the potential for leveraging side-effects as therapeutic advantages. Treatment initiation with SSRIs may be accompanied by an initial worsening of anxiety and irritability in the first

few weeks. Long-term side effects can include gastrointestinal bleeding, weight gain, hyponatremia, and sexual dysfunction. Additionally, nausea, diarrhea, vomiting, and bloating are common gastrointestinal effects that may arise due to the high density of serotonin receptors in the gut.[85]

Citalopram was first developed in 1971 from the norepinephrine reuptake inhibitor talopram.[105] Citalopram is a racemic mix of both R and S enantiomers of citalopram, where the S enantiomer is the compound of interest when treating depression, while the R enantiomer appears to have no effect and may even interfere with the effects of its racemate.[106] As with other SSRIs, citalopram's mechanism of action hinges on competitively inhibiting SLC6A4, leading to elevated extracellular serotonin levels and subsequent interactions with various serotonin receptor subtypes. As previously described, the initial rise in serotonin concentration may lead to a desensitization of presynaptic $5HT_{1A}$ autoreceptors, potentially disinhibiting serotonergic neuronal firing and contributing to downstream mood regulation. Postsynaptic $5HT_{1A}$ receptors are also thought to be involved in the antidepressant response. Additionally, the involvement of $5HT_{2A/C}$ in citalopram's action is a topic of ongoing debate, with some studies suggesting their potential contribution to therapeutic effects and others indicating minimal influence.[107,108]

Fluoxetine holds the distinction of being the first and perhaps the most extensively studied SSRI. It was initially approved as a drug for medical use in Belgium in 1986 and then approved by the FDA in 1987, marketed under brand names like Prozac® and Sarafem®. Like citalopram, fluoxetine is also composed of both, R and S enantiomers. However, both enantiomers offer equal potency as inhibitors of the serotonin transporter.[109]

Compared to other SSRIs, fluoxetine demonstrates a lower degree of selectivity for SLC6A4.[110] However, its binding specificity remains significantly higher compared to TCAs and MAOIs. This enhanced selectivity likely contributes to fluoxetine's more favorable side-effect profile. Consequently, at higher doses, it may also elevate synaptic concentrations of norepinephrine and dopamine. Interestingly, some studies have shown that fluoxetine exhibited relatively weak affinity for 5HT receptors, specifically, the $5HTR_1$ and $5HTR_2$ subfamilies.[111–113]

Finally, sertraline, more commonly marketed under the name Zoloft®, a more recent SSRI was approved by the FDA for medical use in the United States in 1991.[114] While more selective than fluoxetine, sertraline exhibits a relatively high affinity for dopamine transporters.[115] Interestingly, sertraline also seems to be more efficacious for the treatment of depression in the acute phase than fluoxetine.[116]

1.2 Cancer and Depression

1.2.1 The Interplay Between Cancer and Depression

Patients with cancer usually present with a variety of somatic symptoms such as fatigue, pain, and loss of appetite.[117,118] These symptoms are further exacerbated by common cancer treatments such as radiotherapy, surgery, and chemotherapy, which, aside from their associated morbidities, place a significant amount of psychological stress on patients.[119–121] This is further supported by several studies that have shown the prevalence of depressive disorders is almost doubled in patients with cancer in comparison to the general population.[13,122,123] In fact, several studies have shown that up to 31% of all cancer patients are diagnosed with major depressive disorder, with its prevalence varying based on the type of cancer, sex, and other socioeconomic factors.[13,124,125] For example, one cross

sectional study found that lung cancer patients having the highest estimated prevalence of depression (13%), followed by gynecological cancers (11%), breast cancer (9%), colorectal cancer (7%), and finally, genitourinary cancers (6%).[126] Of these patients, 27% received any form of effective psychiatric treatment, and only 5% sought care from a mental health professional. Similar to depression as a whole in cancer patients, the prevalence of depression in a specific cancer type can change due to combination of factors including tumor prognosis, pain intensity, body image disruption specific to the cancer type, tumor-induced neuropsychiatric effects, and neuropsychiatric side effects from cancer treatments.

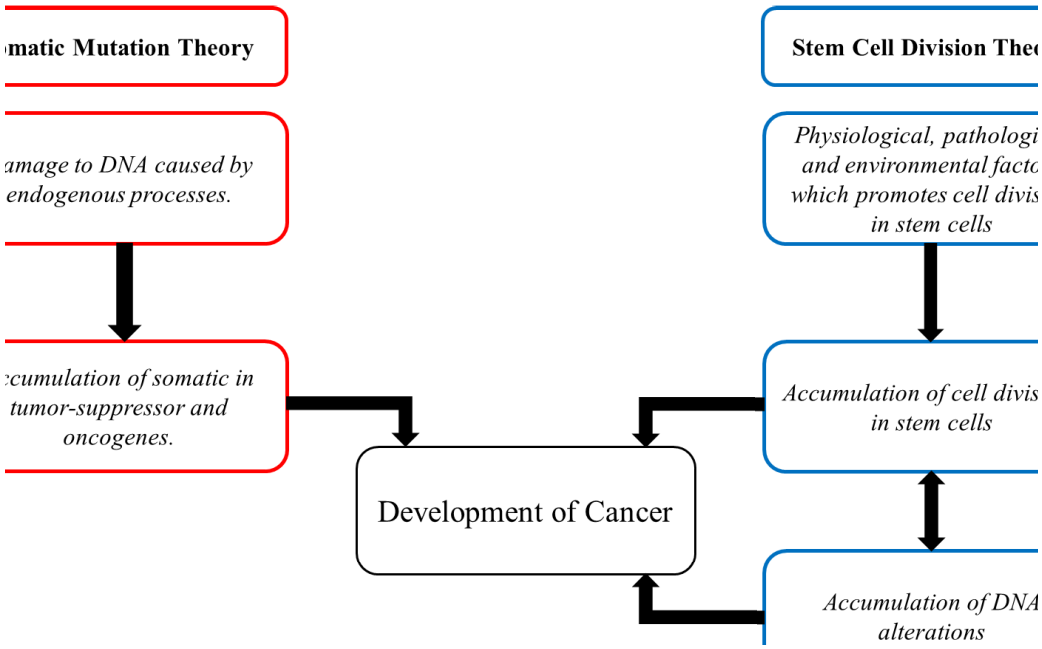
Interpreting depression in cancer patients exclusively as a psychological reaction disregards the role of biological mechanisms and processes in its development. This approach risks minimizing the legitimacy and significance of depression in this context. An early meta-analysis conducted a review of seven prospective longitudinal studies to examine the association between depression and cancer and found that an early history of depression or depressive symptoms predates later onset of cancer.[127] A different study also found a correlation between depression's significant impact on cancer, specifically, patients with cancer were almost 30% more likely to develop depression with about a 34% increase in mortality.[128] While these studies do definitively establish a bilateral connection to the development of cancer and depression, they hint at a deeper connection. Although a direct cause-and-effect relationship remains unclear, the data suggests that the biological processes underlying cancer progression might also initiate depression on a molecular level, potentially even before conscious symptoms arise. The psychological burden of a confirmed diagnosis could then further worsen depressive episodes.[129] Yet, the exact mechanisms linking cancer and depression to each other are not well understood. However,

the role of serotonin, one of the main neurotransmitters involved in the development of depression, plays a significant role in the development of cancer.[10,11,130]

1.2.2 The Role of Serotonin and Serotonin Receptors in Cancer

Cancer is generally classified by the rapid and unregulated proliferation of aberrant cells. However, the underlying cause which leads to the development of cancer is not fully understood. The somatic theory of cancer suggests that specific mutations development into oncogenes which inhibit tumor-suppression genes leading to the development of cancer (Figure 5). These oncogenes result in rapid cell growth while the lack of tumor suppression genes no longer inhibit apoptosis and the arrest of growth during the cell cycle.[131,132] On the other hand, the stem cell theory of cancer takes a slightly different approach when describing which genomic changes lead to the development of cancer (Figure 5). The stem cell theory states that changes in DNA which do not consist of mutations (epigenetic modifications, physiological, pathological, and environmental

factors) but still result in a change in gene expression, lead to the development of cancer.



re 5 The Stem Cell and Somatic Theories of Cancer Development

The n lead to the formation of tumors composed of their differentiated progeny. Conversely, the somatic theory proposes that cancer arises from a gradual accumulation of mutations within mature somatic cells, ultimately disrupting normal growth control and triggering uncontrolled proliferation.

Regardless of the specific etiologies, the defining hallmarks of cancer have generally remained the same. As proposed by Hanahan et al., in 2010 and then updated in 2022, these 10 hallmarks comprise the biological traits which describe the development and progression of cancer, specifically, carcinogenesis and metastasis (Figure 6).[133,134] The first six of which set the foundation of cancer development and progression: (i) stimulation of growth through sustaining proliferative signaling, (ii) insensitivity to inhibitory signals that would inhibit growth and (iii) to signals which would induce apoptosis, (iv) the indefinite proliferation resulting in replicative immortality, (v) the

stimulation of growth of blood vessels to supply nutrients to tumors (angiogenesis), and finally, (vi) the possible invasion of local tissues and metastasis to other locations. The development of these characteristics in abnormal cells is partly due to genetic instability, caused by multiple mutations that initiate and drive tumorigenesis. Additionally, chronic local inflammation associated with various types of cancer also contributes to this process.[135–137] Finally, the last four hallmarks, describe how cancer cells evade detection by the immune system and use altered metabolic pathways.

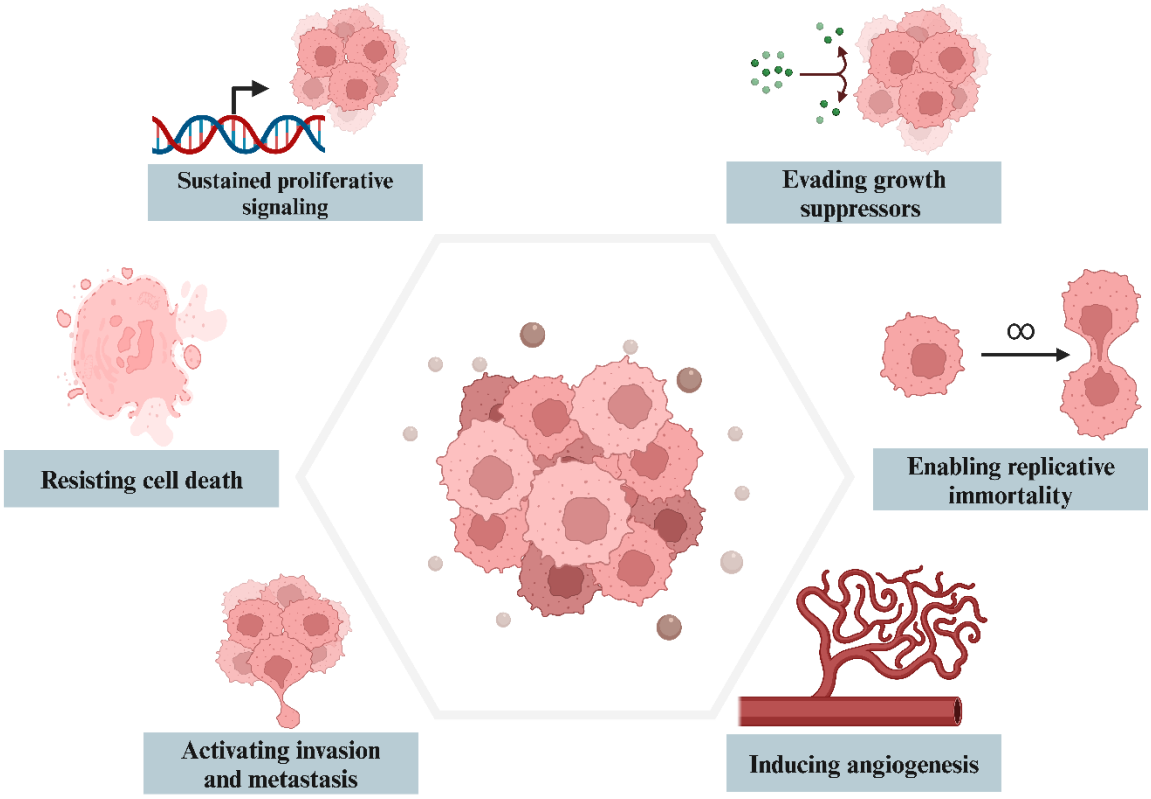


Figure 6 The Hallmarks of Cancer Development

The six core hallmarks of cancer development: sustained proliferative signaling, insensitivity to growth control, evasion of cell death, replicative immortality, promotion of angiogenesis, and the ability to invade local tissues and metastasize. Understanding these hallmarks is critical for developing effective cancer therapies. By targeting these specific capabilities of cancer cells, researchers can aim to disrupt their uncontrolled growth, survival, and spread, ultimately leading to more effective treatment strategies.

While the somatic and stem cell theories of cancer provide a framework for understanding genetic and epigenetic alterations driving tumorigenesis, a deeper comprehension of the microenvironment that fosters these changes is essential. Recent studies unveil a surprising link between serotonin signaling and cancer, suggesting it may play a more multifaceted role than previously appreciated.[10,11] Beyond its well-established influence on depression in cancer patients, serotonin appears to interact with cancer cells and their microenvironment, potentially impacting the hallmarks of cancer as outlined by Hanahan et al., in 2022. These hallmarks encompass critical processes like sustained proliferative signaling, evasion of growth suppressors, replicative immortality, and angiogenesis – all fundamental to tumor development and progression. Elucidating the precise mechanisms by which serotonin modulates these hallmarks could offer a paradigm shift in our understanding of cancer biology. Such insights hold promise for the development of novel therapeutic strategies that target this intricate interplay between the nervous system and the tumor microenvironment, potentially offering a much-needed reprieve for patients battling this multifaceted disease.

Platelets and their activation have emerged as key players in tumor progression and angiogenesis.[138,139] This connection is further strengthened by clinical observations demonstrating an association between thrombocytosis at diagnosis and poorer survival outcomes in various solid tumors. The thrombotic microenvironment characteristic of tumors is known to be a focal point for platelet aggregation, leading to the release of serotonin. This localized surge in serotonin concentration within the tumor microenvironment may represent a novel mechanism contributing to tumor progression and angiogenesis. Notably, these platelet-derived serotonin levels are likely to far exceed

the systemic concentrations observed in healthy individuals.[140] Intriguingly, growing evidence suggests that serotonin production and secretion by neuroendocrine cells contributes to the progression of various solid tumors. This association has been observed in several cancer types, including prostate carcinoma, urinary bladder carcinoma, and small cell lung cancers. The identification of serotonin release from neuroendocrine cells in small cell lung cancer suggests the presence of a potential autocrine loop utilizing serotonin signaling. This loop might play a regulatory role in small cell lung cancer proliferation, thereby presenting a novel mechanism by which neuroendocrine cells could influence tumor growth within the microenvironment.[141] Carcinoid tumors exhibit elevated serotonin secretion, potentially driven by an autocrine loop. This hypothesis aligns with observed 15-20-fold higher blood and tumor tissue serotonin concentration in patients compared to controls.[83] Beyond its localized effects, serotonin's influence extends to the systemic level. In liver metastases, it evades hepatic breakdown, contributing to carcinoid syndrome. Dysregulation of serotonin metabolism observed in cholangiocarcinoma cell lines and biopsies further suggests a potential driver role.[142] Similarly, rising TPH1 expression during breast cancer progression indicates enhanced serotonin biosynthesis, highlighting its broader influence in tumor development across various cancers.[74] These studies suggest that serotonin is a critical player in cancer progression. Beyond localized effects, neuroendocrine cell-derived serotonin may act on neighboring cells through paracrine mechanisms, potentially influencing growth, differentiation, and metastasis.[74] Additionally, findings, coupled with the established role of serotonin and its metabolites as markers in carcinoid tumors, suggest its broader utility as a diagnostic and prognostic tool across various cancers.[143,144] More importantly, however, are the different serotonergic

receptors through which serotonin exerts it carcinogenic effects. Indeed, understanding the role of serotonin in cancer progression reveals an added layer of complexity: tissue-specific receptor responsiveness. This suggests a nuanced model where the biological response within tumors is likely determined by the interplay of multiple serotonin receptor subtypes and their dynamic expression patterns throughout cancer development (Table 1).

In breast cancer, samples derived from both ductal carcinoma and invasive lobular cancer also express multiple serotonin receptor subtypes.[11,145] This study employed a tissue microarray analysis to investigate the expression patterns of serotonin receptors in samples obtained from 102 breast cancer patients. Notably, the analysis revealed inconsistent expression of $5HTR_{1B}$ and $5HTR_{2B}$, with a primarily cytoplasmic localization observed in both cancerous and noncancerous cells. Interestingly, statistically significant associations were identified between $5HTR_{2B}$ expression and the presence of estrogen-α receptors, and between $5HTR_4$ expression and the presence of both estrogen-α and progesterone receptors. However, no significant correlation was observed between serotonin receptor expression and tumor grade. These findings, combined with the observation that elevated tryptophan hydroxylase levels were present in both triple-negative and hormone-dependent cancers, suggests a potential for breast cancer cells to synthesize substantial amounts of serotonin and might be particularly susceptible to the influence of serotonin receptors, warranting further investigation.[74,146,147] For example, the hormone-dependent breast cancer cell line MCF7 has been shown to express $5HTR_{2A,}$ $5HTR_{2C}$, $5HTR_{3A}$, and $5HTR_7$.[148–151] These studies demonstrated that serotonin increased proliferation through $5HTR_{2A}$ and $5HTR_{3A}$, and that the inhibition of these receptors revered that level of proliferation as well as inducing apoptosis. However, triple-negative breast cancer cell

lines, MDA-MB-231, HCC-1395, and Hs 578T, exhibited an even greater expression of Tryptophan hydroxylase and therefore citalopram a greater response to serotonin through $5HTR_7$, when compared to hormone-dependent cell lines.[74] It does seem that in both triple-negative and hormone-dependent breast cancer cell lines, specifically MCF7 and MDA-MB-231, serotonin inhibited apoptosis by promoting glycolysis and oxidative phosphorylation through the activation of $5HTR_{2A}$ and $5HTR_{2C}$ signaling-mediated extracellular signal-regulated kinase (ERK) 1 and 2, Protein Kinase B phosphorylation, and Hypoxia-inducible factor 1-alpha (HIF1α) expression.[150]

Studies have shown that serum and platelet-derived serotonin levels are elevated in patients with hepatocellular carcinoma.[43,152,153] Similarly, in a study using mice induced with hepatocellular carcinoma, serotonin levels were upregulated, providing further evidence of serotonin's involvement in cancer development and progression.[154] Serotonin's ability to promote cancer development seems to be best attributed to the action of 5HTR receptors. In a different study of 176 patients with liver cancer, an analysis of their tissue samples using immunohistochemical staining, $5HTR_{1B}$ and $5HTR_{2B}$ were found to be expressed in 32% and 35% of the patients, respectively.[155] Additionally, it was shown that both $5HTR_{1B}$ and $5HTR_{2B}$ were associated with an increase in proliferation, whereas the expression of $5HTR_{1B}$ was correlated with tumor grade. In the same study, serotonin was seen to promote cellular survival as well as induce proliferation in Huh7 and HepG2 hepatic cell lines. The western blot analysis suggested that serotonin, through $5HTR_{1B}$ and $5HT_{2B,}$ promoted cancer development, via the phosphorylation of ERK1/2 via the mitogen-activated protein kinase (MAPK) pathway, which is accordance with similar studies.[156] Another study has shown that there was an overexpression of $5HTR_{1D}$, $5HTR_{2B}$ and $5HTR_7$

and a lower expression of $5HTR_{2A}$ and $5HTR_{5A}$ in a study involving 6 cell lines (PLC/PRF/5, MHCC97L, MHCC97H, HuH-7, Hep3B, HepG2) and 33 pairs of tumor and adjacent non-tumor liver tissues were obtained from patients, using qPCR.[157] Finally, tissue microarray and immunohistochemistry data gathered from liver cancer patients have shown that the expression of $5HTR_{1B}$ and $5HTR_{2B}$ are associated with cell proliferation index, and that $5HTR_{1B}$ expression is associated with tumor grade.[158,159]

Significantly higher serum serotonin levels have also been observed in patients with lung cancer and depression as compared to patients without depression[160] In this study, tissues from 64 lung adenocarcinoma patients with depression and 64 lung adenocarcinoma patients without depression were evaluated for the expression of 5HT receptors using immunohistochemistry and fluorescence-activated cell sorting. It was found that the expression of $5HTR_{1A}$ and $5HTR_{1B}$ in tumor tissues were higher in patients with depression. Furthermore, it was confirmed that elevated levels of serotonin increased the expression of serotonin receptors and decreased overall survival in lung cancer patients with depression compared to those without depression. Similarly, in a different study, mice deprived of peripheral serotonin exhibited significantly reduced tumor growth.[161] Additionally, it was shown that $5HTR_{1A}$ and $5HTR_{1D}$, played a role in the development of small cell lung cancer.[141,162] Recently, a bioluminescence-based cell viability assay was developed to identify potential pharmacological treatments for small cell lung cancer. Using the Library of Pharmacologically Active Compounds (LOPAC), a database which comprises 1,280 pharmacological agents, only twelve compounds specifically targeting the serotonin signaling pathways were found to decrease small cell lung cancer cell viability. These compounds included a $5HTR_{1B}$ receptor agonist (CGS12066A) and antagonist

(SB224289), a $5HTR_2$ receptor antagonist (ritanserin), a $5HTR_{2B/2C}$ antagonist (SB228357), a $5HTR_{1D/2}$ antagonist (metergoline), a $5HTR_{1B/1D}$ antagonist (GR127935), and selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine, paroxetine, and sertraline. These results continue to display the oncogenic effects of the $5HTR_1$ and $5HTR_2$ family of receptors.

Different subtypes of serotonin receptors are found in both androgen-sensitive (PC3, DU145) and androgen-insensitive (LNCaP & hPCP) prostate cancer cell lines. These serotonin receptor subtypes are present at the original tumor locations as well as at sites of metastasis in human patients.[163–165] Serotonin exhibits a more pronounced proliferative impact on androgen-insensitive prostate cancer cells compared to androgen-sensitive ones. It exerts its growth-promoting effects on prostate cancer through the activation of the $5HTR_{1A}$, $5HTR_{1B}$, $5HTR_{2B}$, and $5HTR_4$ receptors. Specifically, serotonin influences prostate cancer cell growth by modulating MAPK, Phosphoinositide 3-kinases (PI3K), and Protein Kinase B (Akt) signaling pathways. In the PC3 prostate cancer cell line, serotonin triggers the phosphorylation of ERK1/2 via the MAPK pathway, an action that is likely facilitated through $5HTR_{1A}$ signaling, although the involvement of other receptor subtypes has not been entirely excluded. Furthermore, serotonin enhances the phosphorylation of Akt and activates the PI3K/Akt pathway in the DU-145 prostate cancer cell line. This activation of the Akt pathway by serotonin also plays a role in inhibiting apoptosis in prostate cancer cell lines, potentially through mechanisms involving $5HTR_{1B}$ signaling. These findings indicate that serotonin may play a role in the advancement of prostate cancer. Nonetheless, it is important to note that the specific modulation of the PI3K/Akt pathway by serotonin in PC3 and LNCaP prostate cancer cell lines remains uncertain, given

these cells' inherent activation of the PI3K/Akt pathway due to the loss of the PTEN tumor suppressor gene.[166,167]

In colorectal cancer, serotonin regulates both cancer cell proliferation and angiogenesis, contributing to the overall advancement of tumors. Elevated serum serotonin levels in individuals with colon cancer indicate a potential association, again, highlighting serotonin as a potential prognostic marker for gauging the progression of colon cancer.[168] Several studies have shown, through western blotting and immunohistochemistry, that the colon cancer cell line HT29 expressed serotonin receptors $5HTR_{1A}$, $5HTR_{1B}$, $5HTR_{1D,}$ $5HTR_3$ and $5HTR_3$.[45,169] Studies by the same authors showed that serotonin inhibits apoptosis as well as having a slight mitogenic effect on HT29 cells, via the activation of the $5HTR_{1A}$, $5HTR_{1B}$, $5HTR_{3,}$ and $5HTR_4$ receptors.[45,169,170] However, studies done on mice colon cancer cell lines, CT26 and MC38, demonstrated that a deficiency in peripheral serotonin decreases the growth of tumors in mouse models implanted with colon cancer allografts. Moreover, the administration of external serotonin was shown to reverse this condition.[161] However, this does not seem to be based on serotonin's impact as a result of its direct influence on cancer cells. Instead, serotonin promotes angiogenesis by reducing the expression of matrix metalloproteinase 12 (MMP12) in tumor-infiltrating macrophages. MMP12 plays a regulatory role in angiogenesis by elevating circulating levels of angiostatin, a potent inhibitor of blood vessel formation. In the context of colon cancer, serotonin plays a role in regulating both cancer cell proliferation and angiogenesis, ultimately contributing to the progression of tumors.[168] Similarly, a more recent studies found that increased levels of serotonin lead to the activation of lymphocytes directing to cytokine release, which mirrors human inflammatory bowel disease, and that an

intraperitoneal injection of serotonin had a mitogenic effect in the descending colon of adenocarcinoma cells in rats.[171]

It has been shown that in pancreatic ductal adenocarcinoma cells lines AsPC-1, BxPC-3, Capan-2, CFPAC-1, HPAC, PANC-1, SW1990PANC-1 and MIAPaCa-2, and in several mouse models that $4HT_{1B}$, $5HTR_{1D}$, and $5HTR_{2B}$ were overexpressed as compared to normal pancreas tissues, while the expression of other 5HTR subtypes were undetectable.[172,173]. Indeed, the authors found that serotonin, as seen in other cancer types, increased cell viability and inhibited apoptosis, likely due to the stimulation of glycolysis and increased expression of HIF1α and myelocytomatosis oncogene (c-MYC). They also observed that $5HTR_{2B}$ was in part responsible through its activation of the (PI3Ks)/mammalian target of rapamycin(mTOR)/Protein Kinase B (Akt) pathways. In support of this, they showed that the antagonism of this receptor was shown to decrease cancer growth and inhibited migration. A different study using the pancreatic cancer cell line PaCa, demonstrated that, $5HTR_{1B}$, and $5HTR_{1D}$ promoted tumor growth and stimulated metastasis.[173] The actions of $5HTR_{1B}$ and $5HTR_{1D}$ signaling is mediated through Src-focal adhesion kinase (FAK) and the transglutaminase 2 modulation of nuclear factor-κB (NF-κB) signaling axis.[173]

While not particularly well studied, urinary bladder cancer seems to have the most currently known serotonin receptors. In an early study, using 35 patient prostatic tissues, $5HTR_{1A}$ and $5HTR_{1B}$ were found overexpressed, especially in high grade tumors.[174] Another study found that $5HTR_{1A}$, $5HTR_{1B}$, $5HTR_{1D}$, $5HTR_{2B}$, and $5HTR_{2c}$ were expressed in human bladder cancer tissues and in the HT1376 cell line.[164,175]

Finally, it is pertinent to speak on the effects of serotonin receptors in one of the most severe forms of cancer, glioblastoma. This form of cancer, above all gives credence to the role of depression and the effects of serotonin on cancer development. Not only because serotonin is most often thought to mainly act in the brain to modulate mood, but because depression is especially associated with a poor prognosis in glioblastoma patients.[176] A study using several proteomic methods revealed that $5HTR_7$ was expressed in the human glioblastoma cell lines U-373 MG, U-138 MG, U-87 MG, DBTRG-05MG, T98G, H4, CCF-STTG1 and Hs 683.[177] This study further strengthen the proposed role of 5-hydroxytryptamine receptor 7 (5-HTR7), a separate study demonstrated that serotonin treatment stimulates the secretion of interleukin-6 (IL-6) in U-373 MG astrocytoma cells. This stimulatory effect was mediated by 5-HTR7 signaling through the p38 mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) pathways. Supporting the involvement of $5HTR_7$ another study found that serotonin treatment promoted interleukin-6 (IL-6) secretion in U-373 MG astrocytoma cells.[178] This stimulatory effect was mediated through 5-HTR7-dependent activation of the p38 MAPK and PKC signaling pathways. A different study, using LN229 and U251 MG human glioblastoma cell lines, demonstrated a lower expression of $5HTR_{5A}$ in high-grade glioma than in low-grade glioma.[179] This study demonstrated that the agonization of $5HTR_{5A}$ by valerenic acid effectively inhibited the proliferation in both glioblastoma cell lines and mouse models, but increased intracellular reactive oxygen species levels and activation of 5' adenosine monophosphate-activated protein kinase (AMPK), and subsequently the ability of glioblastoma to migrate.

Cancer type	Models	Serotonin receptor (5HTR) subtypes expressed	References
Breast Cancer	Hormone responsive cell lines (MCF7 and T47D), Triple-negative breast cancer cell lines (MDA-MB-231, HCC-1395, and Hs 578T), and human breast cancer tissues.	1B, 2A, 2B, 2C, 3A, 4, 7	11, 74, 74, 140, 141, 142, 143, 144, 145, 146
Hepatocellular Carcinoma	Human hepatocellular carcinoma cell lines (Huh7 and HepG2), human HCC tissues, and mouse xenograft models.	1D, 1B, 2A, 2B, 5A, 7	43, 147, 148, 149, 150, 151, 152, 153, 154
Lung Cancer	*In vivo* mouse model of Lewis lung cancer and patient lung adenocarcinoma samples with depression,	1A, 1B, 1D, 2B, 2C	138, 155, 156, 157
Prostate Cancer	Prostate cancer cell lines (PC3, DU-145, and LCaNP)	1A, 1B, 2A, 2B, 4	158,159,160,
Colon Cancer	Mice colon cancer cell lines (CT26 and MC38), human colon cancer cell line (HT29), and human colon cancer tissue.	1A, 1B, 1D, 3A, 4	42, 156, 161, 162, 163, 164
Pancreatic cancer	Human pancreatic cancer cell lines (AsPC-1, BxPC-3, Capan-2, CFPAC-1, HPAC, PANC-1 and SW1990PANC-1 and MIAPaCa-2) and human pancreatic cancer tissue microarrays.	1B, 1D, 2B	165,166,
Urinary bladder cancer	Human bladder cancer cell line (HT1376) and patient prostatic tissues	1A, 1B, 1D, 2B, 2C	159, 167, 168
Glioblastoma	Glioblastoma cell lines ((U-373 MG, U-138MG, U-87 MG, DBTRG-05MG, T98G, H4, CCF-STTG1 and Hs 683) and Human glioblastoma tissues.	5A, 7	169, 170, 171

Table 1 Serotonin Receptor Expression in Different Cancer Types

The expression of different serotonin receptors in different canter types, and the models in which they were identified.

1.2.3 The Use and Effects of SSRIs in Cancer

As shown, serotonin, through the function of serotonergic receptors, plays an important and complex role in cancer development and progression. The relationship between SSRIs and cancer is equally complex. Recently, SSRIs have garnered increasing attention for their potential role in cancer treatment. Aside from their established ability to alleviate depression and anxiety in cancer patients, SSRIs have demonstrated direct anticancer properties across several cancer types.[180,181] This raises the intriguing possibility

that SSRIs could not only play a role in managing the psychological distress that many cancer patients face, but also simultaneously combat cancer progression. While numerous experiments have demonstrated the anti-cancer properties of SSRIs, some evidence suggests that they might also enhance cancer risk under certain circumstances.[182,183] This highlights the need for a nuanced understanding of the interplay between SSRIs and cancer biology.

Citalopram was shown to trigger apoptosis through a reactive oxygen species-mediated mechanism in hepatocellular carcinoma.[184] This pathway involves the activation of the pro-inflammatory transcription factor NF-κB and the release of cytochrome c from the mitochondria. Besides direct cell death, citalopram also exerts an inhibitory effect on tumor metastasis and invasion in colorectal cancer.[185] This effect is potentially linked to the downregulation of key genes associated with poor prognosis such as BRCA1-Associated Ring Domain protein 1 (BARD1), Myb-related protein B (MYBL2), and baculoviral IAP repeat containing protein (5BIRC5).[186] Furthermore, escitalopram, the S-enantiomer of citalopram, demonstrates anti-proliferative and anti-invasive properties in non-small cell lung cancer cells. Mechanistically, citalopram-induced apoptosis appears to be mediated through caspase-3 activation.[187] These findings collectively suggest a multifaceted role for citalopram in cancer therapy, encompassing the induction of apoptosis, suppression of metastasis, and downregulation of oncogenic factors.[187]

Fluoxetine was shown to be associated with decreased expression of cell cycle regulatory proteins (Cyclin-D1), urokinase-type plasminogen activator (uPA), matrix metallopeptidase-9 (MMP9), and pro-angiogenic factors such as vascular endothelial growth factors (VEGF).[188] In hepatocellular carcinoma and non-small cell lung cancer,

fluoxetine has been shown to inhibit pro-survival pathways, potentially through the downregulation of the AKT/NF-κB or ERK /NF-κB signaling cascades. A similar study found that in non-small cell lung cancer, fluoxetine disrupts cell cycle control, triggering a coordinated response involving autophagy and cell cycle arrest.[189] This effect is mediated by the Activating transcription factor 4 (ATF4)/Akt/mTOR signaling pathway. Fluoxetine directly activates ATF4, which then acts to suppress the pro-survival Akt-mTOR pathway. fluoxetine's anti-cancer properties extend to colon cancer.[190] By elevating the nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκB-α) and of kappa light polypeptide gene enhancer in B-cells inhibitor beta (IκB-β), which inhibit the pro-inflammatory transcription factor NF-κB, fluoxetine can effectively silence NF-κB, leading to a downstream decrease in the expression of like VEGF and c-Myc, both of which are crucial for colon cancer cell proliferation.[191] Fluoxetine's anti-tumor repertoire extends further than canonical signaling pathways. One study suggest it can induce apoptosis through calcium-mediated mechanisms involving the endoplasmic reticulum (ER) and mitochondria.[192] Fluoxetine disrupts mitochondrial function, leading to decreased ATP generation and a subsequent depletion of calcium stores within the ER. This disruption in calcium homeostasis can further trigger an accumulation of calcium and iron within the mitochondria, ultimately pushing tumor cells towards late-stage apoptosis. Additionally, fluoxetine has been shown to induce calcium influx while concurrently decreasing anti-apoptotic protein Bcl-2.[193] This combined effect disrupts mitochondrial membrane potential, promoting DNA cleavage and apoptosis in Burkitt's lymphoma cells. These findings highlight a unique and multifaceted strategy employed by fluoxetine to eliminate cancer cells.

Sertraline has demonstrated unexpected potential as an anticancer drug, as it exhibits direct anti-tumor effects and possesses the intriguing ability to overcome multidrug resistance, a major hurdle in cancer treatment.[194] One mechanism by which sertraline circumvents drug resistance is through the inhibition of drug efflux pumps, effectively blocking cancer cells from expelling chemotherapeutic agents.[195] Furthermore, sertraline targets the PI3K/Akt/mTOR signaling cascade, a well-known pathway that promotes cell survival and proliferation. By inhibiting this pathway, sertraline disrupts a key pro-survival mechanism in cancer cells, ultimately leading to cell death.[196–198] Sertraline's anti-tumor activity extends beyond established signaling pathways. Studies suggest it can directly target translationally controlled tumor protein (TCTP), a critical factor for the survival of tumor stem cells. Inhibition of TCTP by sertraline effectively induces tumor cell death. Mechanistically, sertraline appears to induce a form of cell death known as autophagy, a self-degradative process. This process involves the generation of free radicals, including elevated levels of hydrogen peroxide and peroxidative lipids, alongside a reduction in the antioxidant glutathione.[199] Additionally, sertraline downregulates key autophagy markers such as light chain 3 (LC3), ATG5, and Beclin 1. Interestingly, sertraline also appears to target stem cell and epithelial-mesenchymal transition markers, potentially impacting tumor cell plasticity and metastasis. The anti-tumor effects of sertraline are further amplified when combined with other drugs. For instance, studies have shown a synergistic effect on anti-proliferation when sertraline is used alongside fluoxetine. Sertraline can also enhance the apoptotic effects of tumor necrosis factor-related apoptosis inducing ligand. This occurs through the activation of the AMPK cascade, leading to an increase in death receptor 5 expression and ultimately promoting apoptosis in cancer cells.[200]

Collectively, these findings suggest a broader role for SSRIs beyond their current use in treating depression and anxiety. The intricate dance between the serotonergic system and cancer biology is further illuminated. Serotonin, acting through its diverse receptors, exerts a complex and multifaceted influence on cancer development and progression. The relationship between SSRIs and cancer is equally intriguing. Recent research suggests a potential for SSRIs to directly target cancer cells across various types. This prospect raises the possibility that SSRIs could not only alleviate the emotional burden of cancer patients but also simultaneously combat cancer progression itself. However, some evidence suggests a potential increase in cancer risk under specific circumstances, highlighting the need for a more nuanced understanding. Further research is crucial to delineate the precise mechanisms underlying the observed anti-cancer effects of SSRIs and identify patient populations who would benefit most from this promising therapeutic approach.

1.3 Experimental Design

Building on the established link between depression and cancer progression, this study delves deeper to explore the complex interplay between serotonin signaling and cancer development. While the detrimental influence of depression on cancer development and progression is well-documented, the precise mechanisms by which serotonin and its receptors influence tumorigenesis remain elusive. This study aimed to shed light on this critical gap in knowledge by examining the categorization of various serotonin receptors expressed in diverse cancer types and their combined effects on cancer development and progression.

The initial stage involved the selection of a panel of cell lines encompassing prevalent and well-characterized human cancers. Informed by the existing scientific literature, we

opted for a diverse range of cell lines to ensure generalizability. This selection includes A549 cells, reflecting the high incidence and mortality rate associated with lung cancer. MCF7 cells to model the hormone-dependent breast cancer subtypes frequently linked to depression, as breast cancer represents the most commonly diagnosed cancer globally. Hs 578T and MDA-MB-231 cell lines broaden the study's scope by encompassing the aggressive triple-negative breast cancer subtype. Finally, SK-N-AS cells were selected to acknowledge the traditional connection between serotonin and the central nervous system, where neuroblastoma originates. As shown in Table 1, several of the chosen cell lines, possess partially characterized serotonin receptor profiles, allowing us to leverage existing knowledge and establish a robust foundation for further exploration.

The second phase entailed the selection of a variety of serotonin receptors for in-depth examination. We focused on receptors $5HTR_{1B}$, $5HTR_{1D}$, $5HTR_{1E}$, $5HTR_{2A}$, $5HTR_{2B}$, $5HTR_{2C}$, $5HTR_{5A}$, and $5HTR_{7,}$ as well as the serotonin transporter. These receptors were chosen based on a combination of several factors: their established characterization in the literature, commercial availability of antibodies, and the application profile of the antibodies such as use in western blotting, flow cytometry, and immunofluorescent imaging. This baseline expression data served as a crucial foundation for subsequent phases of our research. By measuring the expression levels of these receptors in our chosen cell lines, we can effectively gauge their response to serotonin or SSRI treatment. Changes in serotonin receptor expression following these treatments will provide valuable insights into the functional role of these receptors in various biological processes. Understanding the baseline expression profile is essential for interpreting the magnitude and direction of changes observed after treatment. Ultimately, this approach allows us to decipher the

intricate interplay between serotonin signaling and cellular function. This comprehensive approach will equip us to investigate a broad spectrum of serotonin signaling pathways potentially implicated in cancer progression in future studies.

Building on the established serotonin receptor expression profile, the next phase refines our experimental design. To determine optimal concentrations for serotonin and SSRI treatments, an MTT assay, a colorimetric assay used to measure cell viability was performed to determine the concentrations used to perform subsequent experiments. These concentrations represent their IC_{20} values, the concentration at which they decrease viability by 20%, which demonstrate each treatment maximal biological activity without being attributed to toxicity or antiproliferative effects. These optimized concentrations were then used for various assays. First each cell line was treated with serotonin and each SSRI to determine how serotonin receptor expression levels are affected. Concurrently, the effects of serotonin and SSRIs on cell growth and migration were also examined in each cell line.

By employing this multifaceted investigative approach, the study sought to illuminate the complex role of serotonin signaling in cancer biology. The findings will contribute significantly to a deeper understanding of the mechanisms by which depression and 5HT influence tumor development and progression. This knowledge has the potential to pave the way for the development of novel therapeutic strategies that target both the psychological and biological aspects of cancer, offering a more holistic approach to patient care.

2 Materials and Methods

2.1 Antibodies and Reagents

Serotonin (B21263-03) and selective serotonin reuptake inhibitors (SSRIs): citalopram hydrobromide (AC462310010), fluoxetine hydrochloride (AAJ61197MF), and sertraline hydrochloride (AC462190010) were purchased from Thermo Fisher Scientific Inc (Waltham, MA, USA). Antibodies directed against $5HTR_7$ (01675704), $5HTR_{1B}$ (PA5-65031), $5HTR_{1D}$ (501981897), $5HTR_{1E}$ (PIPA597847), $5HTR_{2A}$ (501981900), $5HTR_{2B}$ (720256), $5HTR_{2C}$ (72 026 4), $5HTR_{5A}$ (50 1981906), SLC6A4 (702076), and Goat anti-Rabbit IgG Secondary Antibody Alexa Fluor™ 488 (A-11008), SuperBlock™ Blocking Buffer (37515) were obtained from Thermo Fisher Scientific Inc (Waltham, MA, USA). Toxicity and growth assays were evaluated through measurement of mitochondrial dehydrogenase activities with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Sigma-Aldrich, St. Louis, MO, USA) and total cellular protein content through the measurement of Sulforhodamine B sodium salt (SRB) from Thermo Fisher Scientific Inc. VECTASHIELD® Hardset™ Antifade Mounting Medium with DAPI (H-1500) was acquired from Vector Laboratories (Burlingame, CA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, and sodium pyruvate were obtained from Thermo Fisher Scientific Inc (Waltham, MA, USA).

2.2 Cell Culture

A549 (ATCC® CCL-185), Hs 578T (ATCC® HTB-126), MCF7 (ATCC® HTB-22TM), MDA-MB-231 (ATCC® HTB-26), and SK-N-AS (ATCC® CRL-2137) (ATCC, Manassas, VA), cells were grown in DMEM supplemented with 10% (v/v) FBS, 100 IU/ml

penicillin, 100 µg/ml streptomycin and 1.0 mM sodium pyruvate. All cell lines were maintained on tissue culture plastic substrate and kept at 37°C in a humidified atmosphere containing 5-10% CO_2.

2.3 In vitro cytotoxicity assay

Cell viability was tested in accordance with Romijn et al.[201] Briefly, the activity of mitochondrial dehydrogenase was measured by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide reagent (MTT). Cells were seeded in 96-well plates at an initial density of 1.5 x 10^4 cells in 100 µl of the appropriate culture medium. Following an overnight incubation, cells were treated with 0.1 µM or 1 µM of 5HT of serotonin, and 1 µM, 5 µM, or 10 µM of each SSRI. After 24 and 48 hours, 100 µl of medium was removed prior to the addition of MTT. The formed formazan crystals were then dissolved in 200 µl dimethyl-sulfoxide (DMSO) (Thermo Fischer Scientific). Eight wells were used for each condition and concentration across at least three independent experiments to determine the mean optical density (OD), reflecting cell viability, using a Cytation™ 1 Cell Imaging Multi-mode reader with Gen5 software (Agilent Technologies Inc., Santa Clara, CA). IC_{20} values were expressed as percentages as compared to solvent treated control cells.

2.4 In vitro cell growth assay

Cells were seeded in 96-well plates at an initial density of 1.5 x 10^4 cells in 100 µl of the appropriate culture medium. Following an overnight incubation, cells were treated with 1 µM of 5HT, 10 µM of each SSRI, and then with a combination of 1 µM of 5HT and 10 µM of each SSRI. Following a 12 and 24 hours incubation period, the amount of cell protein in each well was estimated with the Sulforhodamine B assay in accordance with

Orellana et al.[202] Eight wells were used for each condition and concentration across at least three independent experiments to determine the mean optical density (OD), reflecting cell growth, using a Cytation™ 1 Cell Imaging Multi-mode reader with Gen5 software (Agilent Technologies Inc., Santa Clara, CA). Using the optimized and most physiologically relevant concentrations derived from the MTT assay, the percentage of growth inhibition was then determined as compared compared to solvent treated controls.

2.5 In vitro wound-healing assay

The effects of serotonin and SSRIs on migration was evaluated using a scratch (wound healing) assay. In brief, cells were grown in 96-well Corning plates until appropriately confluent (~90%). An automatic scratch was performed in the middle of the wells using the AutoScratch™ Wound Making Tool (Agilent Technologies Inc., Santa Clara, CA). Following wound formation, the media was removed, and each well was washed as recommended by the manufacturer. After the wells were washed, they were treated with serotonin and SSRIs at the concentrations determined from previous MTT assays. Representative images (4x magnification) were recorded immediately after the scratch was performed to represent the 0 hour time point, and after 12, 24 and 48 hours using the Cytation™ 1 Cell Imaging Multi-mode reader and the Scratch Assay App for masking and wound width analysis. Using the Scratch Assay App wound width, or the average width of the cell free zone over time was calculated using the following equation:

$$Wt = \frac{I_A - Object\ Sum\ Area_t}{I_H}$$

Where Wt is the average wound width (µm) over time, I_A is the total area of the 4x image, Object Sum Area is the area covered by cells at each time point, and I_H is the height of a 4x image.

2.6 Flow cytometric analysis.

Following treatments with serotonin and SSRIs at each corresponding timepoint, cells were detached and suspended as single cells using 10 mM Ethylenediaminetetraacetic acid (EDTA). The EDTA was neutralized with phosphate-buffered saline (PBS) and washed again with the appropriate medium containing 0.1% bovine serum albumin (BSA). Samples were incubated with the relevant primary 5HTR antibodies, followed by a secondary Alexa Fluor™ 488 antibody. After washing, samples were analyzed with the Attune NxT Flow Cytometer and corresponding software (Thermo Fisher Scientific).

2.7 Immunofluorescence imaging

Cells were grown on glass cover slips (diameter, 12 mm) and placed in 24-well plates. Covers slips were treated with serotonin and SSRIs at the concentrations determined from previous MTT assays. Following treatments, glass cover slips were removed, washed, and fixed with 4% paraformaldehyde. Next, fixed cells were washed, permeabilized with 0.1% Triton X-100, blocked with blocking buffer, and then incubated with each 5HTR antibody followed by an incubation with Alexa Fluor™ 488 secondary antibody. Stained cells were mounted with Vectashield® mounting medium. Control staining's were performed without primary antibodies.

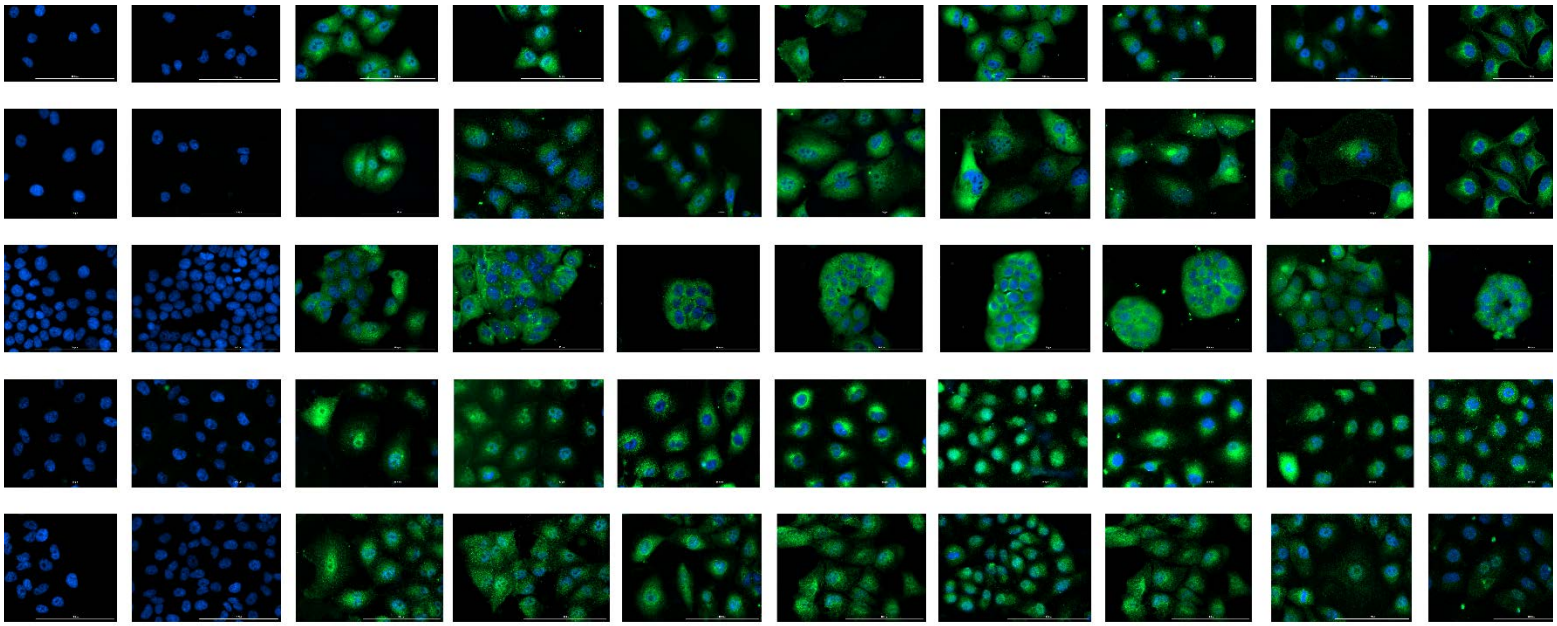
2.8 Statistics

All treatments were matched and performed at least 3 times. Data were analyzed using Excel, for determination of mean, standard deviation (SD) and Student's *t*-test with 95% confidence intervals ($CI_{95\%}$).

3 Results

3.1 The Initial Characterization of Serotonin Receptors

To establish a baseline serotonin receptor expression profile, this initial phase of the study focused on determining the expression of serotonin receptors $5HTR_{1B}$, $5HTR_{1D}$, $5HTR_{1E}$, $5HTR_{2A}$, $5HTR_{2B}$, $5HTR_{2C}$, $5HTR_{5A}$, and $5HTR_{7}$ and the serotonin transporter (SLC6A4) in a panel of cell lines including A549 lung and SK-N-AS neuroblastoma cell lines as well as the hormone-sensitive MCF7 breast cancer and triple-negative breast cancer cell lines Hs 578T and MDA-MB-231. To achieve this, we employed immunofluorescence imaging, followed by flow cytometry to confirm the presence of each serotonin receptor and the serotonin transporter. The immunofluorescence imaging results revealed that $5HTR_{1B}$, $5HTR_{1D}$, $5HTR_{1E}$, $5HTR_{2A}$, $5HTR_{2B}$, $5HTR_{2C}$, $5HTR_{5A}$, and $5HTR_{7}$, and SLC6A4 were present in all the cell lines (Figure 7). These findings were further corroborated with flow cytometry, which consistently confirmed the expression of these receptors and the transporter across each cell line tested (Figures 13-18). It should be noted however that $5HTR_{1B}$ was not expressed in either immunofluorescence imaging studies or through a flow cytometric analysis.



Figur

A baseline serotonin receptor expression profile for $5HTR_{1B}$, $5HTR_{1D}$, $5HTR_{1E}$, $5HTR_{2A}$, $5HTR_{2B}$, $5HTR_{2C}$, $5HTR_{5A}$, $5HTR_{7}$, and the serotonin transporter (SLC6A4) was characterized using fluorescent microscopy in A549, Hs 578T, MCF7, MDA-MB-231 and SK-N-AS.

3.2 The Effect of Serotonin and SSRIs on Cell Viability

Building on the established link between free serotonin as a potential marker for recurrent or metastatic cancer, the effects of 5HT and three SSRIs (citalopram, fluoxetine, and sertraline) on cell viability were examined on three breast cancer cell lines (MCF7, Hs 578T, MDA-MB-231), a lung cancer cell line (A549), and a brain cancer line (SK-N-AS).[143,144] This was done to derive their IC_{20} values, the concentration at which these components decrease viability by 20%, which demonstrates the maximal biological activity of each treatment without the effect being attributed to toxicity. Each cell line was treated with 0.1 μM and 1 μM of 5HT, which represent a range of clinically typical serum concentrations for 24 and 48 hours.[203] Additionally, each cell line was also treated with 1 μM, 5 μM, and 10 μM of each SSRI for 24 and 48 hours to determine their effect on cell viability as compared to solvent treated controls. The results of which are expressed as means relevant to solvent treated controls as shown in Table 2.

Our findings revealed a generally modest cytotoxic effect of 5HT across all tested cell lines (Figure 7). In the A549 lung cancer cell line, treatment with 0.1 μM 5HT caused no significant changes in cell viability after 24 hours and a slight decrease in cell viability of 8% was observed after 48 hours. Additionally, treatment with 1 μM of 5HT induced a decrease in cell viability of 10% after 24 hours and 17% after 48 hours, respectively. Similar 0.1 μM and 1 μM 5HT treatment had minimal effects on the cell viability of the triple-negative breast cancer cell line Hs 578T after 24 hours and a decrease of 8% was observed after 48 hours with the highest concentration, 1 μM of 5HT, tested. In the estrogen- sensitive MCF7 breast cancer cell line, 0.1 μM of 5HT decreased the cell viability

by 7% after 24 and 48 hours. The 24 hours treatment with 1 μM of 5HT did not exhibit any significant effects on the cell viability and a slight reduction of 5% was observed after 48 hours. No significant changes in cell viability were seen after 24 and 48 hours in the more aggressive and highly invasive triple-negative breast cancer model MDA-MB-231 when 0.1 μM of serotonin was added and in the presence of 1 μM the viability was reduced with 6% after 48 hours. Lastly, in the SK-N-AS neuroblastoma cell line, 0.1 μM and 1 μM of 5HT caused a decrease of cell viability of 5% and 12% respectively, while interestingly no significant changes were detected after 48 hours.

The effects of the SSRIs citalopram, fluoxetine, and sertraline on the cell viability and this in physiologically relevant concentrations of 1 μM, 5 μM, and 10 μM, were also evaluated after 24 and 48 hours. The SSRIs displayed varying degrees of cytotoxicity in a dose- and time-dependent manner in all cell lines (Figure 8). In the A549 lung cancer cell line, all three SSRIs exhibited a decrease in cell viability with increasing concentrations. Citalopram induced a modest decrease in cell viability. At 1 μM, the cell viability was reduced by 13% after 24 hours, and by 20% after 48 hours. The effects of the higher concentrations, 5 μM and 10 μM, were very similar with a decrease of 16% and 15% after 24 hours and 10% and 15% after 48 hours. Fluoxetine had a similar cytotoxic effect on A549 cells. More specifically, treatment with 1 μM of fluoxetine caused a 10% decrease in cell viability after 24 hours and further diminished to 13% after 48 hours. In the presence of 5 μM, the cell viability was affected by 13% after 24 hours and 12% after 48 hours. At the highest concentration of 10 μM, fluoxetine caused an 18% reduction in cell viability after 24 hours, and a slightly lower cytotoxic effect of 15% was observed after 48 hours. Sertraline displayed the strongest cytotoxic activity. At 1 μM, sertraline decreased cell

viability by 18% after 24 hours, and a slightly lower effect of 16% was observed after 48 hours of treatment. The effects of this SSRI, following the addition of 5 μM and 10 μM to the cells for 24 and 48 hours, was very similar and resulted in a 20% reduction in cell viability.

All three SSRIs displayed a dose-dependent effect on the cell viability of the Hs 578T triple-negative breast cancer cells. After 24 hours, 1 μM of citalopram caused a decrease in cell viability of 10%. The cell viability at this concentration was further reduced with almost 17% after 48 hours, while a similar effect was seen for the higher concentrations of 5 μM and 10 μM of citalopram showing reduced effects between 16 and 18%. The effects of fluoxetine on the cell viability were similar to citalopram, with a dose-dependent decrease in cell viability. These decreases ranged from 7% at 1 μM to 15% at 10 μM after 24 hours. After 24 hours, treatments with 1 μM, 5 μM and 10 μM of fluoxetine decreased cell viability by 7%, 14% and 15%, respectively. Also, in the Hs 578T triple-negative breast cancer cells, sertraline exhibited the strongest cytotoxic activity. Here, treatment with 1 μM of sertraline caused a decrease in cell viability of 12% after 24 hours, and that effect was even more pronounced after 48 hours with a reduction in cell viability of 21%. Similarly, 24 and 48 hours of sertraline treatments reduced the cell viability with 15% and 22%, and 26% and 30%, for the respective concentrations of 5 μM and 10 μM.

In the hormone-sensitive MCF7 cell line, the 24 hours citalopram treatment showed a decrease in cell viability, which was reduced by 16 and 17% at 1 μM and 5 μM. respectively and was almost 20% at 10 μM. These effects remained relatively consistent when the cells were grown in the presence of citalopram for 48 hours at all test concentrations. Next, very similar though slightly more toxic results were found for

fluoxetine and this for the 24 and 48 hours treatment periods. Namely, a decrease in cell viability of 18% at a concentration of 1 μM, 21-23% at 5 μM, and 22-23% at 10 μM. In line with the above-described results on A549 lung and Hs 578T triple-negative breast cancer cells, sertraline exhibited the most potent and a fairly dose-dependent effect on the MCF7 cell viability. After the treatment of 24 hours with 1 μM, 5 μM, and 10 μM of sertraline, the cell viability was reduced with 23%, 25%, and 23%, respectively. These effects of sertraline were further intensified to 27% at 1 μM, 28% at 5 μM, and 28% at 10 μM after 48 hours of treatment.

Next, the effect of the SSRIs on the viability of the more aggressive triple-negative MDA-MB-231 breast cancer cells showed an interesting pattern: at the lowest treatment concentration, 1 μM, of all the SSRIs, the effect seemed to be less pronounced as compared to the other cell lines in our panel, while the highest concentration, 10 μM, showed a nearly similar toxicity profile after 48 hours. That is, citalopram exerted minimal effects on cell viability at 1 μM after 24 and 48 hours, and results of the 5 and 10 μM treatments showed a gradual increase in toxic effect, reducing the cell viability with an average of 10% and 15% after 24 hours and 48 hours. The effects of the 24 hours fluoxetine treatment on cell viability were minimal for both the 1 and 5 μM concentrations, while a reduction of 12% was seen for the highest concentration of 10 μM. After 48 hours of treatment, the effects of fluoxetine on the cell viability for all tested concentrations followed a dose-dependent pattern and was in a range of 10 to 15%. Treatment with 1 μM of sertraline caused a decrease of 10% at 24 hours, and then 19% at 48 hours. Additionally, treatments with 5 μM and 10 μM of sertraline, resulted in a more pronounced decrease, ranging from about 9% to 16% after 24 hours and increasing to about 23% after the 48 hours treatment period.

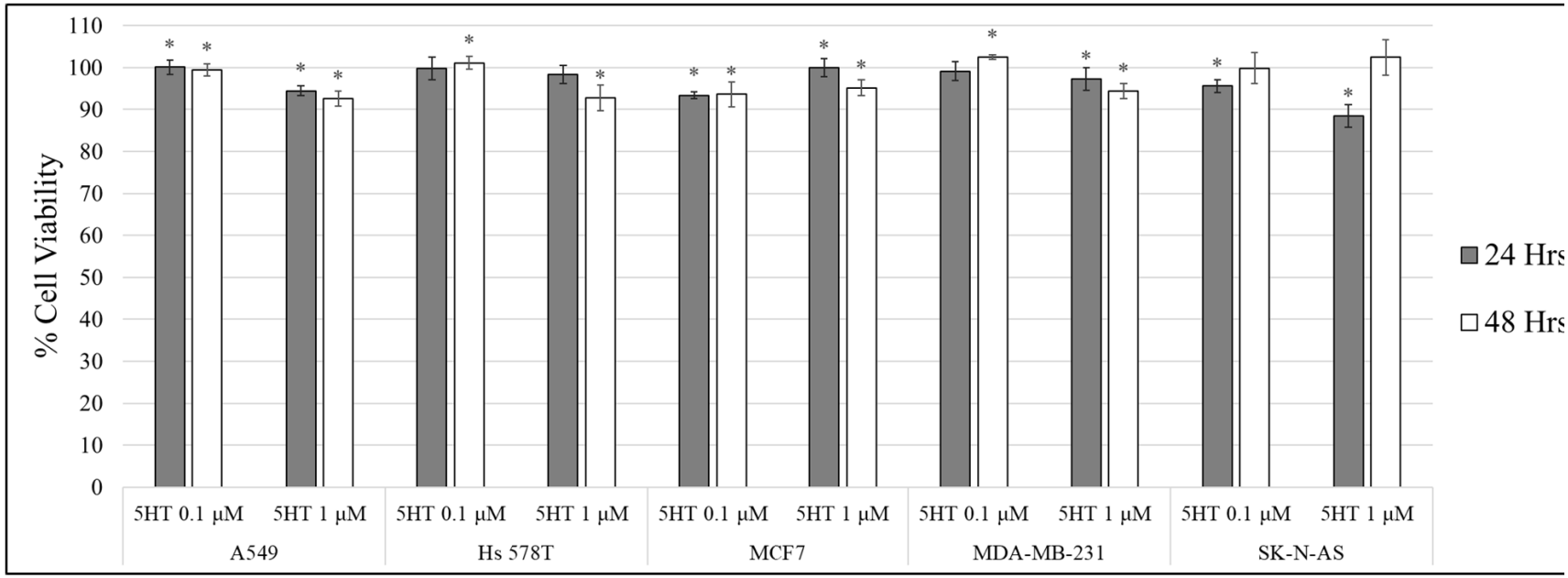
Finally, the SSRI treatments of SK-N-AS neuroblastoma cells exhibited a similar cytotoxic effect or a transient cytotoxic effect after 24 and 48 hours treatments. Citalopram caused a decrease in cell viability ranging from 7% at 1 μM, 9% at 5 μM to 15% at 10 μM after 24 hours. This effect was very similar when the cells were treated for 48 hours. In contrast, results of the effects of fluoxetine and sertraline treatments on the cell viability after 24 were more pronounced than after 48 hours. More specifically, 24 hours of fluoxetine treatment reduced the cell viability with 11%, 14% and 15% for the respective concentrations of 1, 5 and 10 μM, while the effect was only 7% or up to almost 10% for the highest concentrations after the 48 hours treatment period. Interestingly, sertraline also demonstrated a transient and dose-dependent cytotoxic effect. After 24 hours, in the presence of 1 μM and 5 μM of sertraline the cell viability decreased by 16% and by 22% at a concentration of 10 μM. The cytotoxic effects of sertraline were diminished after 48 hours, where 1 μM, 5 μM, and 10 μM of sertraline only decreased the cell viability by 10%, 15%, and 19%, respectively.

	A549		Hs 578T		MCF7		MDA-MB-231		SK-N-AS	
	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs
5HT 0.1 µM	100.1±1.72	92.39±1.43	99.75±2.7	101.14±1.5	93.4±0.85	93.65±2.97	99.12±3.21	102.46±0.5	95.62±1.5	99.84±3.71
5HT 1 µM	90.47±1.09	83.64±1.78	98.34±3.17	92.77±3.13	99.89±2.15	95.15±1.88	97.33±2.7	94.39±1.73	88.44±2.74	102.43±4.22
Cit 1 µM	86.95±1.98	80.98±2.53	90.18±3.49	83.43±1.12	84.45±2.23	83.79±1.7	95.03±1.72	96.79±3.04	92.75±1.57	94.76±1.35
Cit 5 µM	84.04±2.41	90.16±2.33	85.21±2.83	82±3.84	83.03±1.31	84.2±2.71	90.14±2.8	95.35±3.99	91.12±1.12	92.79±0.48
Cit 10 µM	85.49±1.83	84.91±1.99	84.82±2.98	84.53±0.79	81.45±1.89	82.05±0.14	85.83±2.7	83.28±4.78	85.25±0.9	85.54±2.21
Flux 1 µM	89.78±2.68	86.54±1.9	92.58±1.21	89.18±1.45	81.56±2.85	82.24±1.74	95.9±0.89	90.08±4.36	89.04±1.7	92.85±0.49
Flux 5 µM	86.92±2.65	87.56±2.35	86.43±1.42	84.85±0.46	76.57±0.87	79.07±0.26	95.11±0.58	89.44±3.77	85.98±1.24	90.53±1.69
Flux 10 µM	81.57±0.45	84.82±1.83	84.75±1.64	85.61±1.39	77.84±2.12	76.53±2.17	87.82±3.31	85.72±3.51	84.66±0.65	90.6±2.72
Sert 1 µM	82.24±0.72	83.82±1.41	88.41±0.71	79.12±3.6	77.15±1.96	73.33±2.85	90.18±3.87	81.09±2.6	84.12±1.59	89.84±3.27
Sert 5 µM	80.89±1.21	80.45±1.46	85.06±0.29	77.51±1.83	74.94±2.06	71.95±0.12	91.01±2.33	76.87±2.38	83.11±1.88	84.9±3.03
Sert 10 µM	79.99±2.04	79.81±1.7	73.87±2.7	71.35±1.88	77.38±2.21	72.05±2.94	84.01±2.15	78.5±2.25	77.45±2.89	80.85±1.24

The cytotoxic effects of 5HT and SSRIs on A549, Hs 578T, MCF7, MDA-MB-231, and SK-N-AS as determined by an MTT assay. Results are expressed as means ± SD of at least three independent experiments.

	12 hrs	24 hrs	12 hrs	24 hrs	12 hrs	24 hrs	12 hrs	24 hrs	12 hrs	24 hrs
5HT	107.86±2.52	106±2.09	99.14±2.62	97.33±0.51	112.41±3.39	114.9±5.68	97.98±2.97	104.42±6.16	99.44±3	102.88±1.12
Cit	117.33±1.67	104.01±1.33	107.63±3.26	95.64±1.85	120.74±4.43	134.75±2.9	101.96±4.99	103.23±3.24	116.25±4.71	103.82±3.18
Flux	116.69±2.83	109.32±2.67	92.78±3.44	102.95±3.82	102.14±1.18	127.01±2.01	101.99±4.39	113.23±5.9	93.81±4.23	99.66±2.05
Sert	115.17±2.72	106.03±1.98	103.86±2.72	93.61±2.41	117.56±5.96	134.37±3.86	114.36±3.94	105.02±4.58	112.38±1.96	94.07±0.51
Cit+5HT	115.38±2.56	105.95±2.31	93.84±3.86	102.07±2.7	106.79±2.14	121.43±5.68	103.28±3.8	119.86±2.58	97.76±4.57	101.67±2.8

Table 3 The Effects of Serotonin and SSRIs on Cell Growth.



*Bar graphs depict relative cell viability assessed by MTT of A549, Hs 578T, MCF7, MDA-MB-231 and SK-N-AS cells in response to treatment with 0.1 µM or 1 µM of 5HT over 24 hrs (grey) and 48 hrs (white). Data are depicted as means ± STDEV and normalized to solvent treated controls. * Denotes a statistically significant difference from solvent treated control ($p<0.05$).*

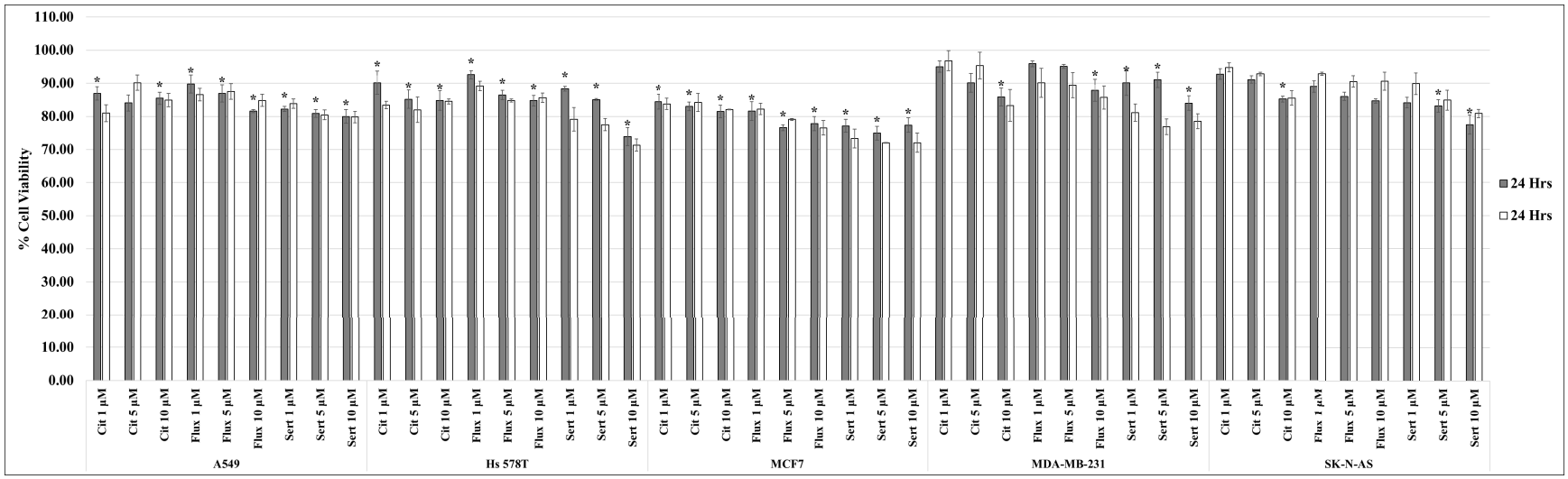


Figure 9 The Effects of SSRIs on Cell Viability

*Bar graphs depict relative cell viability assessed by MTT of A549, Hs 578T, MCF7, MDA-MB-231 and SK-N-AS cells in response to treatment with 1 µM, 5 µM and 10 µM of each SSRI over 24 hrs (grey) and 48 hrs (white). Data are depicted as mean ± STDEV and normalized to solvent treated controls. * Denotes a statistically significant difference from solvent treated control ($p<0.05$).*

3.3 The Effects of Serotonin and SSRIs in Cell Growth

Using the concentrations derived from the previous MTT experiments, 5HT and each SSRI were evaluated for their possible effect on cell growth. Each cell line was treated with 1 μM of 5HT and 10 μM of each SSRI. Additionally, each cell line was treated with each SSRI and 5HT in combination to determine how they affected cell growth together. These findings revealed intricate interactions between 5HT and SSRIs on cell growth, varying based on the specific cell line and treatment combination. Detailed results of which are expressed as means relevant to solvent treated controls are shown in Table 3.

In the A549 lung cancer cells, 5HT increased cell growth by 8% after 12 hours and 6% after 24 hours (Figure 9). 5HT treatment had the least effect on the triple-negative cell line Hs 578T and showed no significant changes in cell growth after 12 hours but decreased cell growth by 3% after 24 hours. However, 5HT had the greatest effect on the estrogen-sensitive MCF7 breast cancer cells, where relative growth was increased by 12% after 12 hours and by 15% after 24 hours. In the triple-negative breast cancer cell line MDA-MB-231, 5HT treatment showed a negligible decrease in cell growth of 2% after 12 hours and then an increase of 4% after 24 hours. Similarly, 5HT treatment only resulted in minor changes in the neuroblastoma cell line SK-N-AS, with a negligible increase of 3% after 24 hours.

However, there seemed to be a more varied response to SSRI treatment, with relative cell growth decreasing over time when treated with each SSRI, except for the estrogen-sensitive cell line, MCF7 (Figure 10). In the lung cancer cell line A549, citalopram treatment led to a significant increase in cell growth of 17% and 4% after 12

and 24 hours, respectively. Fluoxetine showed a similar trend, with increases of 17% after 12 hours and 9% after 24 hours. Sertraline also enhanced growth, with increases of 15% and 6% after 12 and 24 hours. SSRI treatment resulted in minimal effects on the triple-negative breast cancer cell line Hs 578T. Citalopram increased growth by 8% at 12 hours but led to a decrease of 4% after 24 hours. Fluoxetine initially decreased growth by 7% but then resulted in a marginal increase of about 3% at 24 hours. Sertraline's effects were very similar, with an increase of 4% at 12 hours and then a decrease of 6% after 24 hours. The estrogen-sensitive breast cancer cell line MCF7 responded strongly when treated with citalopram, where growth was increased by 20% and 35% after 12 and 24 hours, respectively. While fluoxetine initially increased cell growth by 2% after 12 hours, this increase rose to 27% after 24 hours. Sertraline showed increases to growth of 18% and 34% after 12 and 24 hours. In the triple-negative breast cancer cell line MDA-MB-231 citalopram exhibited a negligible decrease in cell growth of 2% after 12 hours and then a decrease of 4% after 24 hours. Fluoxetine increased cell growth by 3% after 12 hours which then increased to 13% after 24 hours. The opposite is seen following treatment with sertraline which initially increased growth by 14% after 12 hours but then only increased growth by 5% after 24 hours. In the neuroblastoma cell line SK-N-AS, citalopram initially increased growth by about 16% after 12 hours which was then reduced to only 4% after 24 hours. Then, fluoxetine initially decreased growth by 6% after 12 hours, but then had no effect after 24 hours. Sertraline increased growth by 12% after 12 hours but then only decreased growth by 6% after 24 hours.

Following the evaluation of the individual effects of 5HT and SSRIs, the combined effects of 5HT and each SSRI were investigated. The results revealed complex interactions,

with responses varying significantly depending on the cell line and specific treatment combination (Figure 10). In the lung cancer cell line A549, combining SSRIs with 5HT yielded initial increases of about 16% after 12 hours for both citalopram+5HT and fluoxetine+5HT treatments. However, this effect was diminished to increases of 6% and 2% after 24 hours, for citalopram+5HT and fluoxetine+5HT, respectively. Sertraline+5HT sustained growth at both 12 and 24 hours, with increases of 12% and 8%, respectively. Conversely, in the triple-negative cell line Hs 578T, the combination treatments showed notable variability. Citalopram+5HT and fluoxetine+5HT decreased growth by about 6% after 12 hours. However, after 24 hours, citalopram+5HT increased growth by 2% while fluoxetine+5HT decreased growth by 12%. Sertraline+5HT caused decreases at both time points, with reductions of 34% and 15% after 12 and 24 hours, respectively. In the estrogen-sensitive breast cancer cell line MCF7, combination treatments maintained elevated growth. Namely, citalopram+5HT increased cell growth by 7% after 12 hours which was then further increased to 21% after 24 hours. Similarly, fluoxetine+5HT increased cell growth by 7% after 12 hours and 24 hours. Treatment with sertraline+5HT resulted in increased growth by 22% at 12 hours but decreased to 5% at 24 hours. For the triple-negative breast cancer cell line MDA-MB-231 cells, combination treatments with citalopram+5HT initially increased cell growth by 3% after 12 hours which then increased to 20% after 24 hours. Then fluoxetine+5HT increased cell growth by 5% after 12 and 24 hours. Sertraline+5HT increased growth by 20% after 12 hours but then showed no significant effect after 24 hours. Finally, for the neuroblastoma cell line SK-N-AS, citalopram+5HT decreased growth by 2% after 12 hours, but then also increased growth by 2% after 24 hours. Then fluoxetine+5HT increased growth by 11% after 12 hours, but

then decreased cell growth by 10% after 24 hours. Sertraline+5HT led to decreases at both time points, with reductions of 3% at 12 hours and 8% at 24 hours.

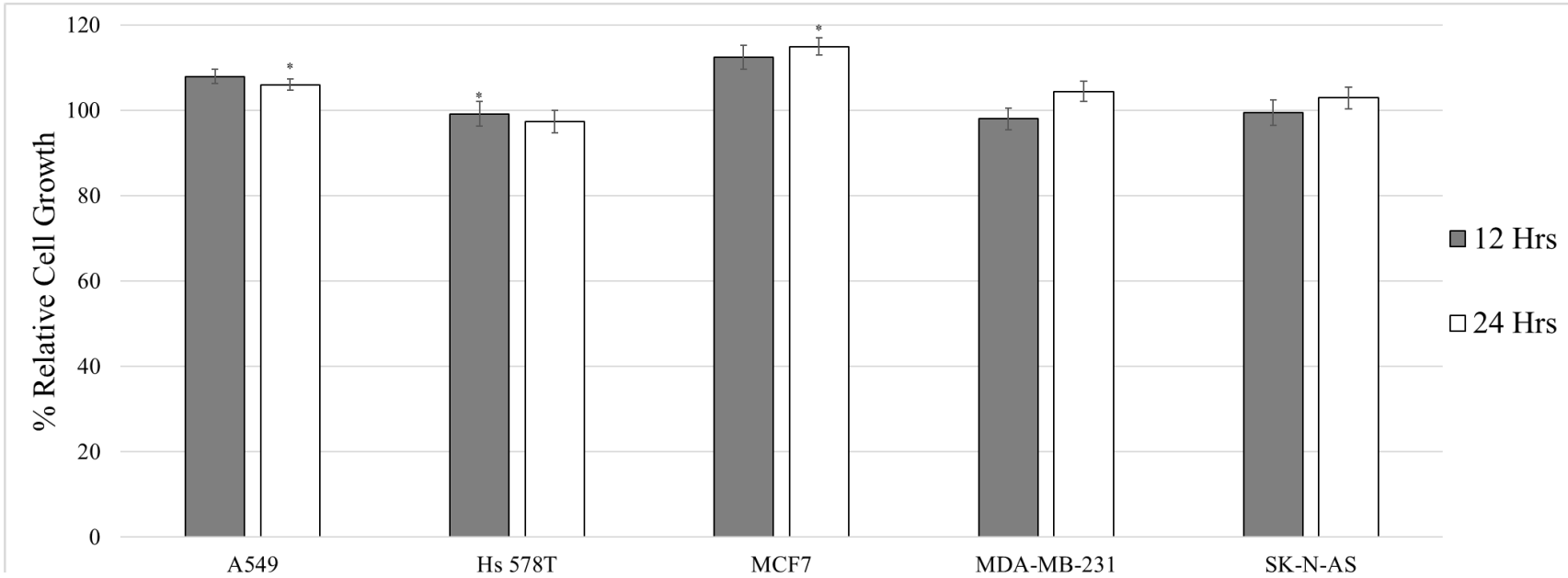


Figure 10 The Effects of Serotonin on Cell Growth

*Bar graphs depict relative growth assessed by an SRB assay of A549, Hs 578T, MCF7, MDA-MB-231 and SK-N-AS cells in response to treatment with 1 μM of 5HT over 12 hrs (grey) and 24 hrs (white). Data are depicted as means ± STDEV and normalized to solvent treated controls. * Denotes a statistically significant difference from solvent treated control ($p<0.05$).*

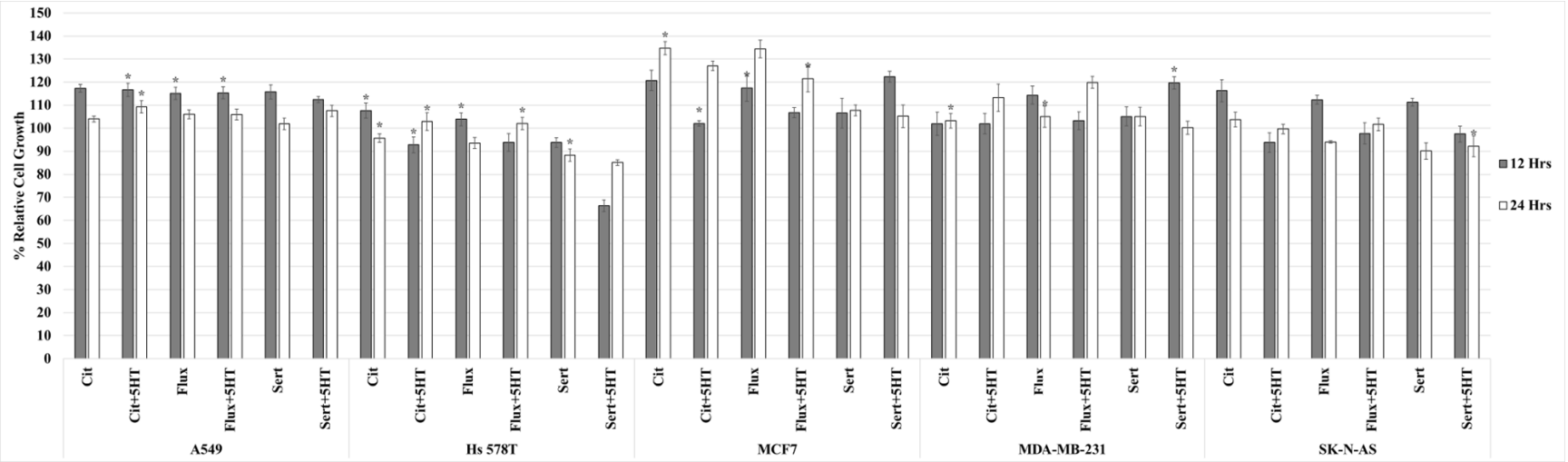


Figure 11 The Combined Effects of SSRIs on Cell Growth.

*Bar graphs depict relative cell growth assessed by an SRB assay of A549, Hs 578T, MCF7, MDA-MB-231 and SK-N-AS cells in response to treatment with 10 μM of each SSRI over 24 hrs (grey) and 48 hrs (white). Data are depicted as means ± STDEV and normalized to solvent treated controls. * Denotes a statistically significant difference from solvent treated control ($p<0.05$).*

3.4 The Impact of Serotonin and SSRIs on Migration

Cancer metastasis is intricately linked to cell growth, as motile cells can readily access distant tissues and establish secondary tumors.[4,5] To better understand how 5HT, SSRIs, and the combined treatments affect a cancer's mobility and metastatic ability, a wound healing assay was performed. Similar to the growth experiments, each cell line was treated with 1 μM of 5HT, 10 μM of each SSRI as well as each SSRI and 5HT in combination, at these concentrations, to determine how they affected migratory velocity. Migratory velocities were assessed at 12, 24 and 48 hours after treatment. Detailed results of the 12 and 24 hours experiments are expressed as relative velocities as compared to solvent treated controls in Table 4. It should also be noted that in the triple-negative breast cancer cell line MDA-MB-231 and in the neuroblastoma cell line SK-N-AS, no data were recorded after 48 hours due to total wound closure after 24 hours.

Generally, there was a dose- and time-dependent effect across most cell lines following 5HT treatment (Figure 12). Initially, in the lung cancer cell line A549, 5HT treatment did not illicit any effect until after 48 hours, where relative migratory velocity was increased by nearly 40%. In the triple-negative breast cancer cell line Hs 578T on the other hand, a constant increase in migratory velocity was observed ranging from 13% at 12 hours, 17% at 24 hours, and 14% at 48 hours when compared to solvent treated controls. In contrast, 5HT treatment of the estrogen-sensitive breast cancer cell line MCF7 did not exhibit the constant effects seen in Hs 578T. After 12 hours, 5HT treatment increased relative migratory velocity by 9%, which was then decreased by 3% after 24 hours with no significant effect observed after 48 hours as compared to solvent treated controls. In the triple-negative breast cancer cell line MDA-MB-231, 5HT increased migratory velocity by

8% after 12 hours, which was then increased to 25% after 24 hours, with total wound closure after 48 hours. The converse was seen in the neuroblastoma cell line SK-N-AS where 5HT increased migratory velocity by 17% after 12 hours and then only increased it by 5% after 24 hours, following total wound closure after 48 hours.

Investigation into the impact of SSRIs on migration revealed diverse responses for each cell line and treatment durations, with citalopram consistently exhibiting the greatest increases and sertraline the greatest decreases to relative migratory (Figure 12). In the lung cancer cell line A549, citalopram exhibited a transient effect in relative migratory velocity with an initial decrease of 15% at 12 hours, which was then increased to 27% at 24 hours, and further increased to 39% at 48 hours. Fluoxetine treatment led to decreases by 30% at 12 hours and 2% at 24 hours, with a significant increase of 43% at 48 hours. Interestingly, sertraline treatment resulted in a significant transient decrease in relative migratory velocity of 58%, 44%, and 38% after 12, 24, and 48 hours, respectively. When the triple-negative breast cancer cells Hs 578T were treated with citalopram, no significant change in relative migratory velocity was seen after 12 hours, but an increase of 11% was observed after 24 hours, and of 15% after 48 hours. Fluoxetine treatment on the other hand resulted in an initial increase of 3% and 4% after 12 and 24 hours, which was then decreased to 21% after 48 hours. However, sertraline treatments led to decreases in relative migratory velocity of about 49%, 59%, and 33% after 12, 24 and 48 hours, respectively. The estrogen-sensitive cell line MCF7 displayed a decrease of about 14% after 12 hours, 20% after 24 hours, and 9% after 48 hours following citalopram treatments. Then following treatment with fluoxetine, the relative migratory velocity was decreased by 9% and 14% after 12 and 24 hours, and increased by 5% after 48 hours. Then sertraline treatments decreased relative

migratory velocity by 42% after 12 hours, while there was no significant change after 24 hours, and interestingly an increase of 21% at 48 hours. In the triple-negative breast cancer cell line MDA-MB-231, citalopram increased relative migratory velocity by 8% after 12 hours, then by 20% after 24 hours with total wound closure after 48 hours. However, fluoxetine only led to an increase in relative migratory velocity of about 8% after 24 hours followed by total would closure after 48 hours. Yet, Sertraline decreased relative migratory velocity by 36% and 23% after 12 and 24 hours, followed by total wound closure after 48 hours. Following treatment of the SK-N-AS neuroblastoma cells with citalopram, an increase in relative migratory velocity of 14% after 12 hours was detected, which then decreased by about 7% after 24 hours, with total wound closure after 48 hours. Fluoxetine increased relative migratory velocity by about 21% after 12 hours, with no significant change after 24 hours, followed by total wound closure after 48 hours. Similarly, sertraline treatments resulted in an increase in relative migratory velocity of about 13% after 12 hours, which was then followed by a 4% decrease in relative migratory value, with total wound closure after 48 hours.

Analysis of the effects of the combined SSRIs and 5HT treatments on migration showed similar effects to SSRI treatments, with sertraline exhibiting consistent and significant decreases to relative migratory velocities (Figure 12). In the A549 lung cancer cell line, treatments with citalopram+5HT showed no significant changes in relative migratory velocity after 12 hours but was then increased to 4% at 24 hours, and a significant increase of 24% after 48 hours. Fluoxetine+5HT treatment resulted in decreases of 4% at 12 hours and 7% at 24 hours, which then reverted to a significant increase of 21% at 48 hours. Sertraline+5HT treatment led to decreases in relative migratory velocity of 64% at

12 hours, 72% at 24 hours, and 72% at 48 hours. The triple-negative breast cancer cell line Hs 578T cells treated with citalopram+5HT exhibited increases of 5% at 12 hours and 11% at 24 hours, followed by an increase of 3% at 48 hours. Fluoxetine+5HT treatment resulted in an increase of 4% after 12 hours, negligible effects after 24 hours, and a decrease of 7% after 48 hours. Sertraline+5HT treatment led to decreases in relative migratory velocity of 64% at 12 and 24 hours, and 72% at 48 hours. When treated with citalopram+5HT, the results in the hormone-sensitive breast cancer cell line MCF7 displayed a substantial increase in relative migratory velocity of 27% at 12 hours, followed by a decrease of 20% at 24 hours, but then a decrease of 10% at 48 hours. Fluoxetine+5HT treatment led to increases in relative migratory velocity of 13% after 12 hours, then a decrease of 11% after 24 hours, and no significant changes after 48 hours. Interestingly, sertraline+5HT treatment resulted in minimal changes in relative migratory velocity, with no significant changes after 12 and 24 hours, but then led to an increase of relative migratory velocity of 29% after 48 hours. Then, in the triple-negative breast cancer cell line MDA-MB-231, citalopram+5HT treatment exhibited an increase of 3% at 12 hours, then a decrease of 3% at 24 hours, with a total wound closure after 48 hours. Fluoxetine+5HT treatment led to an increase of 8% at 12 hours, a decrease of 7% after 24 hours, followed by total wound closure after 48 hours. Sertraline+5HT treatment resulted in decreases in relative migratory velocity of 48% after 12 hours and 25% after 24 hours, with a total wound closure after 48 hours. In the neuroblastoma cell line SK-N-AS, cells treated with citalopram+5HT showed increases of 13% after 12 hours, and no significant effects after 24 hours, with the wound being totally closed after 48 hours. However, fluoxetine+5HT treatment resulted in increases of 13% after 12 hours and a decrease of 8% after 24 hours, with total wound

closure after 48 hours. Finally, sertraline+5HT treatment led to decreases of 8% at 12 hours and then an increase of 6% at 24 hours, with the wound being fully closed after 48 hours.

	A549			Hs 578T			MCF7			MDA-MB-231		SK-N-AS	
	12 hrs	24 hrs	48 hrs	12 hrs	24 hrs	48 hrs	12 hrs	24 hrs	48 hrs	12 hrs	24 hrs	12 hrs	24 hrs
5HT	97.25±5.6	100.95±9.57	139.58±5.29	112.67±5.56	116.81±5.64	113.81±6.71	108.56±6.47	97.07±5.09	101.44±3.49	107.89±5.89	124.65±2.11	117.13±4.14	105.02±5.75
Cit	85.04±3	127.05±1.54	139.11±2.54	99.31±7.39	111.4±3.35	114.93±3.67	85.56±6.54	80.36±3.84	90.72±5.62	100.31±1.28	119.44±3.73	114.42±1.02	93.79±0.45
Flux	70.67±0.28	98.25±2.54	143.1±1.81	103±5.03	104.35±5.29	78.74±5.48	90.65±0.8	85.67±3.75	105.24±4.6	101.85±1.73	108.17±1.98	120.88±1.11	99.65±5.51
Sert	41.78±2.07	55.58±0.73	61.68±5.34	51.44±2.48	40.7±4.32	67.22±1.38	58.35±6.8	99.16±2.64	121.16±5.24	64.11±3.54	77.76±2.27	112.68±5.56	96.33±2.98
Cit+5HT	101.08±4.23	104.27±5.94	124.05±0.33	104.88±7.82	110.94±8.42	103.05±6.43	126.78±3.02	80.44±5.4	89.6±5.24	103.24±2.5	96.55±8.68	113.34±2.83	101.54±6.21
Flux+5HT	96.42±3.82	93.45±5.45	121.39±0.64	103.5±3.35	101.09±2.61	93.15±1.75	113.32±1.91	89.15±1.03	101.38±2.71	108.53±0.61	92.59±4.3	113.48±4.41	92.78±3.08
Sert+5HT	35.98±4.42	28.08±2.4	27.86±0.92	35.07±3.5	36.68±4.24	27.71±2.69	97.81±2.83	99.22±4.68	128.94±3.18	51.76±3.68	75.27±1.61	92.4±6.43	105.71±6.53

Table 4 The Combined Effects of 5HT and SSRIs on Migration

The combined effects of 1 µM 5HT and 10 µM of each SSRI on A549, Hs 578T, MCF7, MDA-MB-231, and SK-N-AS on migration as determined by a wound healing assay. Results are expressed as means ± SD of at least 3 independent experiments.

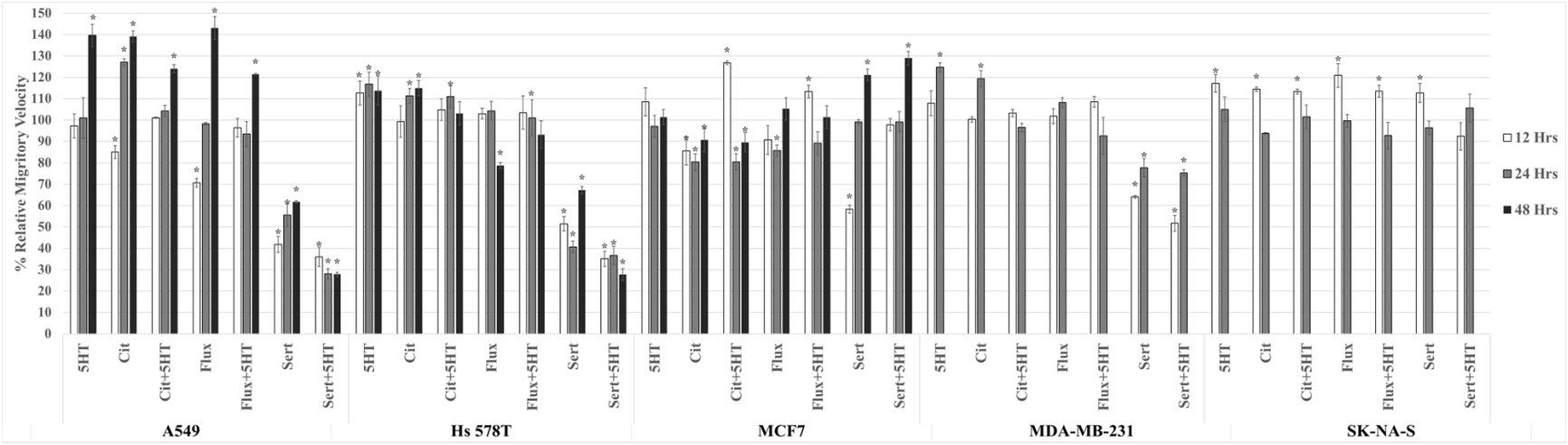


Figure 12 The Combined Effects of 5HT and SSRIs on Migration

Bar graphs depict relative migratory velocities assessed by a wound healing assay of A549, Hs 578T, MCF7, MDA-MB-231 and SK-N-AS cells in response to treatment with combined treatments of 1 µM of 5HT and 10 µM of each SSRI over 12 hrs (white) 24 hrs (grey) and 48 hrs (black). Data are depicted as means ± STDEV and normalized to solvent treated controls. ***** *Denotes a statistically significant difference from solvent treated controls ($p<0.05$).*

3.5 The Characterization of Serotonin Receptors following Serotonin and SSRI Treatment

To further characterize serotonin receptor expression, each cell line was treated with 1 μM of 5HT and 10 μM of each SSRI for 24 and 48 hours. Changes in serotonin receptor expression were analyzed using immunofluorescence microscopy. Then we performed flow cytometry to confirm and quantify the levels of serotonin receptor expression. (Figures 13-18) (Table 5). In the flow cytometry data two color peaks are represented. First a red peak on the left represents cells which were treated with a primary antibody and were used as a control to denote cells which were not expressing serotonin receptors. Then, the black peak represents cells which were treated with a serotonin receptor antibody. The closer the black peak is to the red, the less serotonin receptor expression is observed.

Following 5HT treatment of each cell line, at both 24 and 48 hours, immunofluorescence imaging showed that every receptor except $5HTR_{1B}$ was expressed, with receptor expression characteristics comparable to the baseline expression profile (Figure 13). While the homogenous serotonin receptor expression of the baseline profile is still observed, $5HTR_{2A}$ consistently exhibited areas of serotonin receptor centered around the nucleus in all cell lines except for the triple-negative breast cancer cell line Hs 578T, and the estrogen-sensitive cell line MCF7. Flow cytometry results demonstrated a general rightward shift in the expression of all receptors, except for $5HTR_{1B}$, after 24 hours, indicating an increase in serotonin receptor expression levels (Figures 14-18). However, after 48 hours, receptor expression levels returned to baseline, indicating a transient upregulation in response to serotonin.

Then, each cell line was treated with each SSRI for 24 and 48 hours. Generally, after 24 hours, immunofluorescence microscopy results showed that when treated with SSRIs each cell line did not display the uniform expression pattern of serotonin receptors as seen in the initial characterization and following 5HT treatment (Figures 14-18). The effect on the expression pattern of the serotonin receptors did not seem to change between treatments of 24 and 48 hours. In all cell lines, a distinct clustering of the $5HTR_{2A}$ was observed. However, for $5HTR_{2B}$ there still seems to be a constant level of serotonin receptor expression throughout the cells, but with distinct changes in the $5HTR_{2B}$ expression pattern depending on the cell line. For example, in the triple-negative breast cancer cell line Hs 578T, distinct pockets of $5HTR_{2B}$ clustering were observed following treatments with fluoxetine and sertraline.

Following SSRI treatments, the flow cytometric analysis revealed the presence of two peaks: one peak on the left overlapping with the control, and another peak to the right representing 5HTR expression (Figures 14-18). Generally, the flow cytometric analysis showed a reduction in serotonin receptor expression levels over time with SSRI treatment. In the A549 lung cancer cell line, treatments with citalopram lead to a lower amount of serotonin receptor expression levels while treatments with sertraline resulted in an increase. Following 24 hours of citalopram treatment, there was a constant level of serotonin receptor inhibition as compared to the untreated baseline. The highest level of serotonin receptor expression was for $5HTR_7$ at 32%, while the lowest level was found for the serotonin transporter at 12%. This trend continued after 48 hours, with $5HTR_7$ again being the most expressed at 47%, and the lowest level was recorded for the serotonin transporter and $5HTR_{2A}$ at 25%. Fluoxetine treatment initially did not show a significant effect on the

serotonin receptor expression levels. For example, $5HTR_{1D}$ and $5HTR_{7}$ showed a 58% level of expression as compared to the baseline. Yet, by 48 hours, the expression levels for $5HTR_{1D}$ decreased to 22%, marking the largest decrease. The expression of the serotonin transporter was decreased to 25% and 19% as compared to the baseline after 24 and 48 hours. Sertraline treatment resulted in an increase in serotonin receptor expression levels across all receptors after 24 hours, with the lowest change observed for $5HTR_{2A}$ from 79% to 78% by 48 hours. The serotonin transporter showed an expression level of 88% and 73% as compared to the baseline. For the triple-negative breast cancer cell line Hs 578T cell line, the effects of citalopram and fluoxetine on the expression levels were comparable. Namely, citalopram treatment initially exhibited the highest receptor expression for $5HTR_{2A}$ at 52%, which decreased to 48% after 48 hours. The lowest effect was observed for $5HTR_{2B}$ with 9%, which then increased to 56% after 48 hours. The serotonin transporter initially only showed a 6% expression which then significantly increased to 35% after 48 hours. Following treatments with fluoxetine, the expression of $5HTR_{2B}$ was highest at 55% after 24 hours which was reduced to 12% after 48 hours, showcasing the most significant reduction among treatments. Similarly, $5HTR_{2C}$ and the serotonin transporter exhibit 12% expression as compared to the baseline after 24 hours. Then, $5HTR_{2C}$ expression was increased to 19% and the serotonin transporter was decreased to 9% after 48 hours. Sertraline showed a high initial expression for $5HTR_{2C}$ at 62%, which decreased to 43% after 48 hours, marking the most substantial decrease in this treatment group, since all other receptors exhibited a 47-51% in serotonin receptor expression after 24 hours which then changed to 43-50% after 48 hours. Notable, the serotonin transporter showed a 48% level of expression which decreased to 40% after 48 hours, as compared to the baseline. The

estrogen-sensitive breast cancer cell line MCF7, citalopram had the greatest effect on expression levels while sertraline had the least. Citalopram treatment showed relatively constant serotonin receptor expression levels, with the highest being $5HTR_{2B}$ at 59% after 24 hours, which was then decreased to 55% after 48 hours. The lowest was $5HTR_{2C}$ who's expression level was 39% after 24 hours which then decreased to 36% after 48 hours. The expression levels for the serotonin transporter were relatively constant with 46% expression after 24 hours and 44% after 48 hours as compared to the baseline. Fluoxetine treatment presented the highest initial expressions for $5HTR_{2B}$ at 64% after 24 hours, which was reduced to 62% after 48 hours, while the least initial expression was for $5HTR_{2C}$ at 33% after 24 hours, which then increased to 38% after 48 hours. The expression levels for the serotonin transporter were 46% expression after 24 hours and 48% after 48 hours which was similar to the effects seen following treatment with citalopram. Sertraline treatments resulted in the highest levels of expression for $5HTR_{2B}$ at 30% after 24 hours, increasing to 63% after 48 hours. The smallest changes were noted in $5HTR_{1D}$, moving from 27% to 46% after 24 and 48 hours, respectively. The expression levels of the serotonin transporter were 21% after 24 hours and then nearly doubled to 40% after 48 hours. The triple-negative breast cancer cell line, MDA-MB-231, known for its aggressive nature, demonstrated the highest levels of serotonin receptor expression levels regardless of treatment. Citalopram induced minor fluctuations with the highest expression levels observed in the serotonin transporter at 82% after 24 hours, which increased to 87% after 48 hours, while the lowest was in $5HTR_{2A}$ which went from 74% to 73% after 24 and 48 hours. Following fluoxetine treatment, $5HTR_{2A}$ and $5HTR_{2B}$ exhibited the highest serotonin receptor expression level of 82% after 24 hours which then dropped to 78% and 71% respectively, after 48 hours.

The least amount of serotonin receptor expression was seen in $5HTR_{1E}$ at 7% after 24 hours, increasing to 85% after 48 hours. The serotonin transporter had a 73% expression level after 24 hours which then increased to 84% after 48 hours. Unlike previous treatments, sertraline exhibited the lowest expression levels after 24 hours when then increased to similar levels as seen with citalopram and fluoxetine. After 24 hours, $5HTR_{2B}$ and $5HTR_7$ showed 77% levels of expression which then increased to 70% and 77% after 48 hours, respectively. Overall, all the serotonin transporters saw the highest overall level of expression of 60% after 24 hours but then one of the lowest after 48 hours of 70%. The SK-N-AS neuroblastoma cell line displayed similar trends to the A549 line with sertraline treatment, maintaining high receptor expressions throughout the 24 and 48hour periods. Following treatment with citalopram, $5HTR_7$ exhibited the highest expression level after 24 hours at 36% when then increased to 49% after 48 hours. The serotonin transporter exhibited the lowest total expression levels of 14% after 24 hours and 20% after 48 hours. Similarly, following treatments with fluoxetine, $5HTR_7$ exhibited the highest expression level after 24 hours at 63% when then decreased to 39% after 48 hours. Again, the serotonin transporter exhibited the lowest total expression levels of 29% after 24 hours and 20% after 48 hours. Finally, sertraline treatments in SK-N-AS exhibited the highest levels of expression than any other treat in all cell lines. After 24 hours treatment the serotonin transporter showed the highest level of expression at 92% after 24 hours which then decreased to 77% after 48 hours, as compared to the baseline.

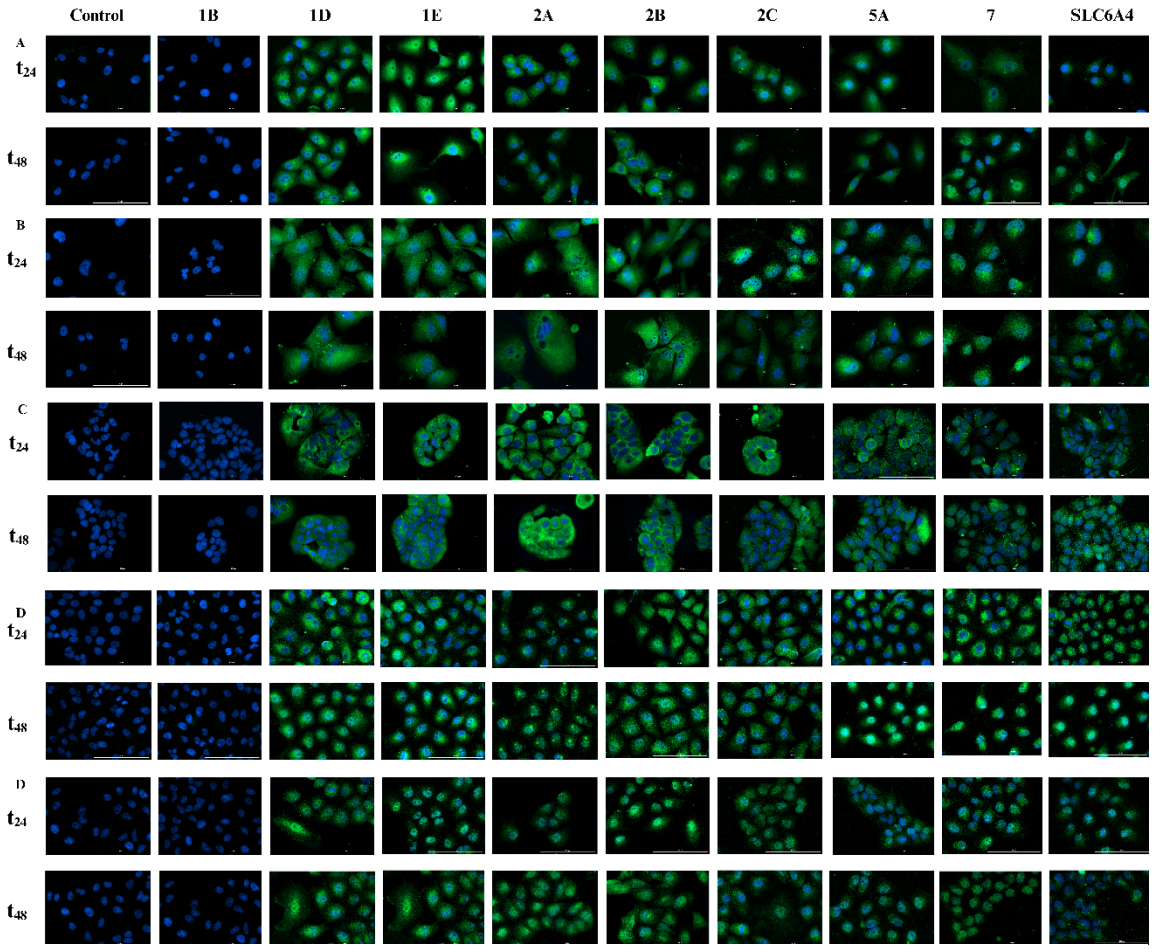


Figure 13 The Effects of Serotonin on the Expression of Serotonin Receptors and the Serotonin Transporter.

A combined expression profile for 5HTR$_{1B}$, 5HTR$_{1D}$, 5HTR$_{1E}$, 5HTR$_{2A}$, 5HTR$_{2B}$, 5HTR$_{2C}$, 5HTR$_{5A}$, and 5HTR$_{7}$, and the serotonin transporter (SLC6A4) was characterized using fluorescent microscopy in (A) A549, (B) Hs 578T, (C) MCF7, (D) MDA-MB-231 and (E) SK-N-AS following 5HT treatment after 24 and 48 hrs.

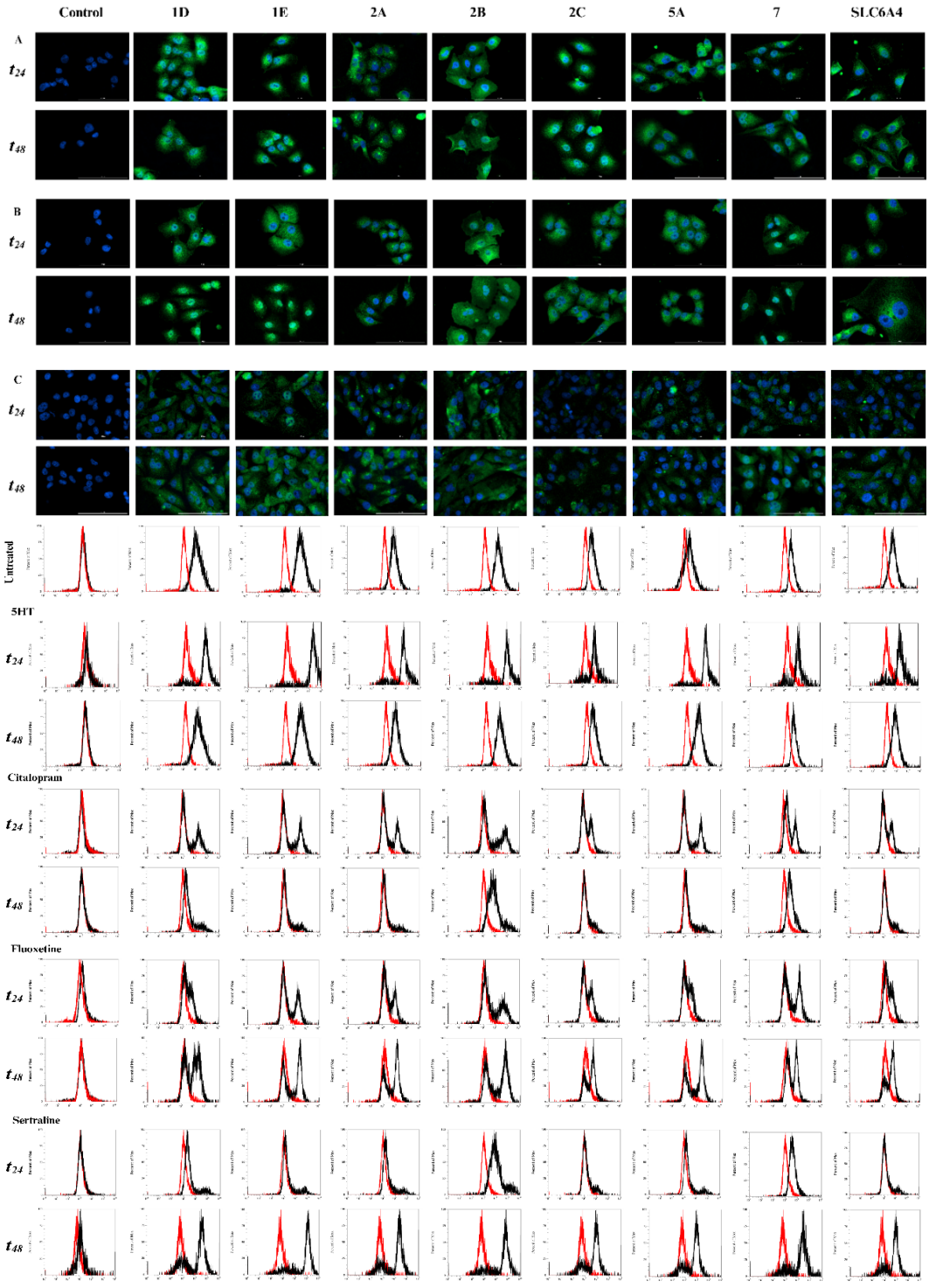


Figure 14 The Combined Effects of 5HT & SSRIs on 5HTR Expression in A549

The combined expression profile for serotonin receptors 5HTR$_{1B}$, 5HTR$_{1D}$, 5HTR$_{1E}$, 5HTR$_{2A}$, 5HTR$_{2B}$, 5HTR$_{2C}$, 5HTR$_{5A}$, and 5HTR$_{7}$, as well as the serotonin transporter (SLC6A4), was characterized using fluorescent microscopy (top) and quantified using flow cytometry (bottom) following 24 and 48 hours of 5HT and SSRI treatments. In the flow cytometry data, two peaks are represented following each treatment: the red peak on the left indicates control cells not incubated with a primary antibody, while the black peak represents cells incubated with a serotonin receptor antibody.

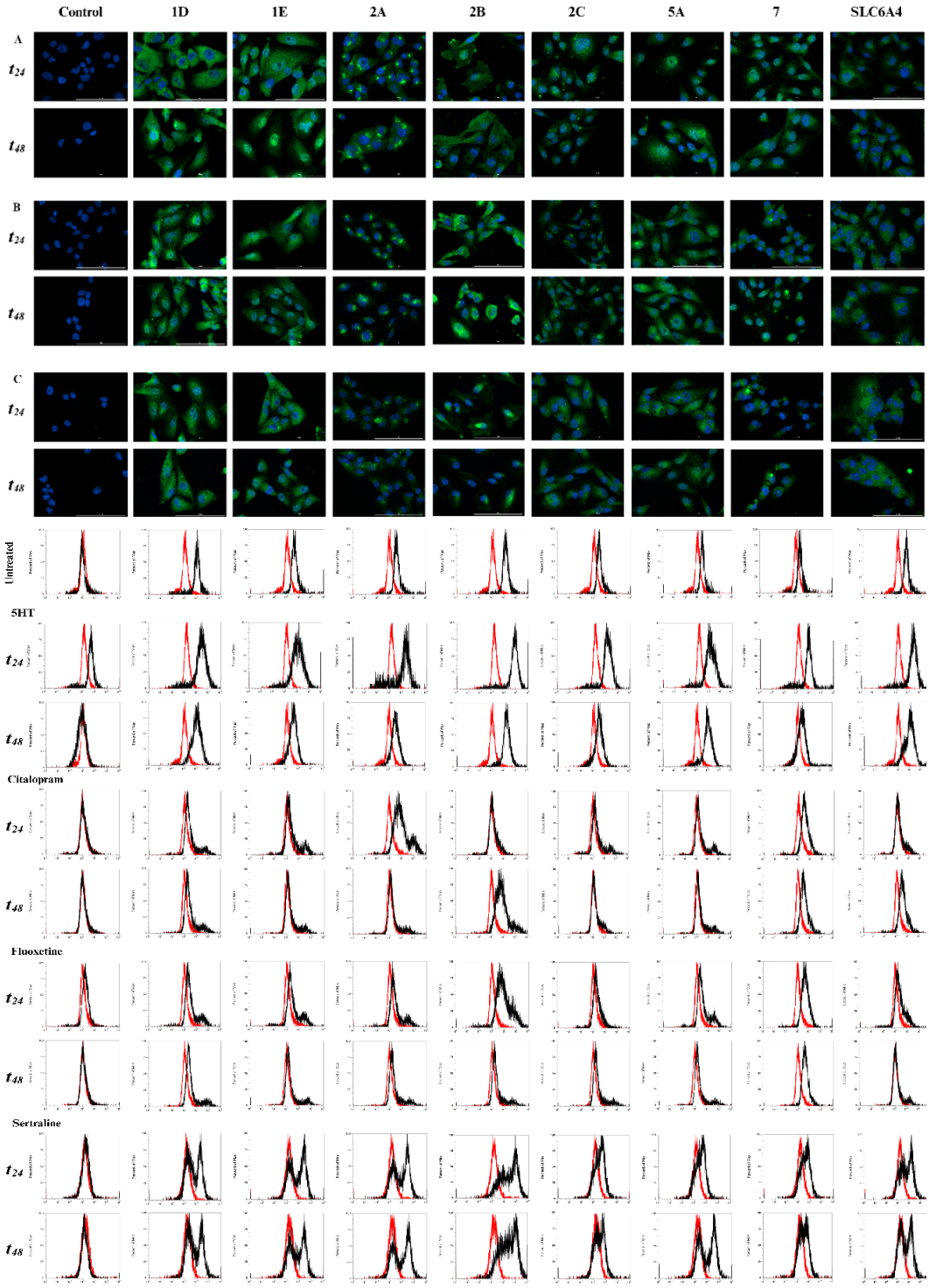


Figure 15 The Combined Effects of 5HT & SSRIs on 5HTR Expression in Hs 578T

The combined expression profile for serotonin receptors $5HTR_{1B}$, $5HTR_{1D}$, $5HTR_{1E}$, $5HTR_{2A}$, $5HTR_{2B}$, $5HTR_{2C}$, $5HTR_{5A}$, and $5HTR_{7}$, as well as the serotonin transporter (SLC6A4), was characterized using fluorescent microscopy (top) and quantified using flow cytometry (bottom) following 24 and 48 hours of 5HT and SSRI treatments. In the flow cytometry data, two peaks are represented following each treatment: the red peak on the left indicates control cells not incubated with a primary antibody, while the black peak represents cells incubated with a serotonin receptor antibody.

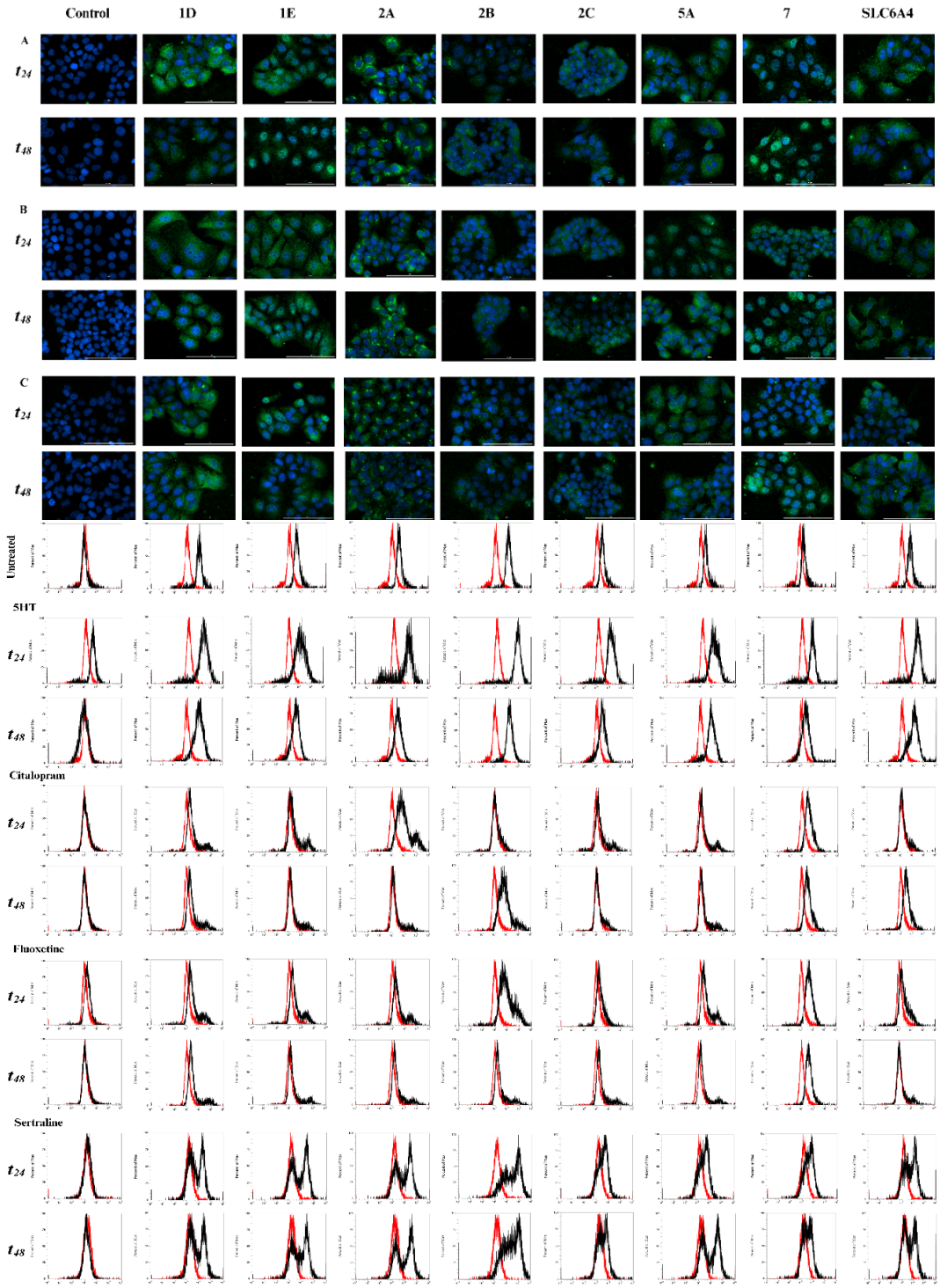


Figure 16 The Combined Effects of 5HT & SSRIs on 5HTR Expression in MCF7

The combined expression profile for serotonin receptors $5HTR_{1B}$, $5HTR_{1D}$, $5HTR_{1E}$, $5HTR_{2A}$, $5HTR_{2B}$, $5HTR_{2C}$, $5HTR_{5A}$, and $5HTR_{7}$, as well as the serotonin transporter (SLC6A4), was characterized using fluorescent microscopy (top) and quantified using flow cytometry (bottom) following 24 and 48 hours of 5HT and SSRI treatments. In the flow cytometry data, two peaks are represented following each treatment: the red peak on the left indicates control cells not incubated with a primary antibody, while the black peak represents cells incubated with a serotonin receptor antibody.

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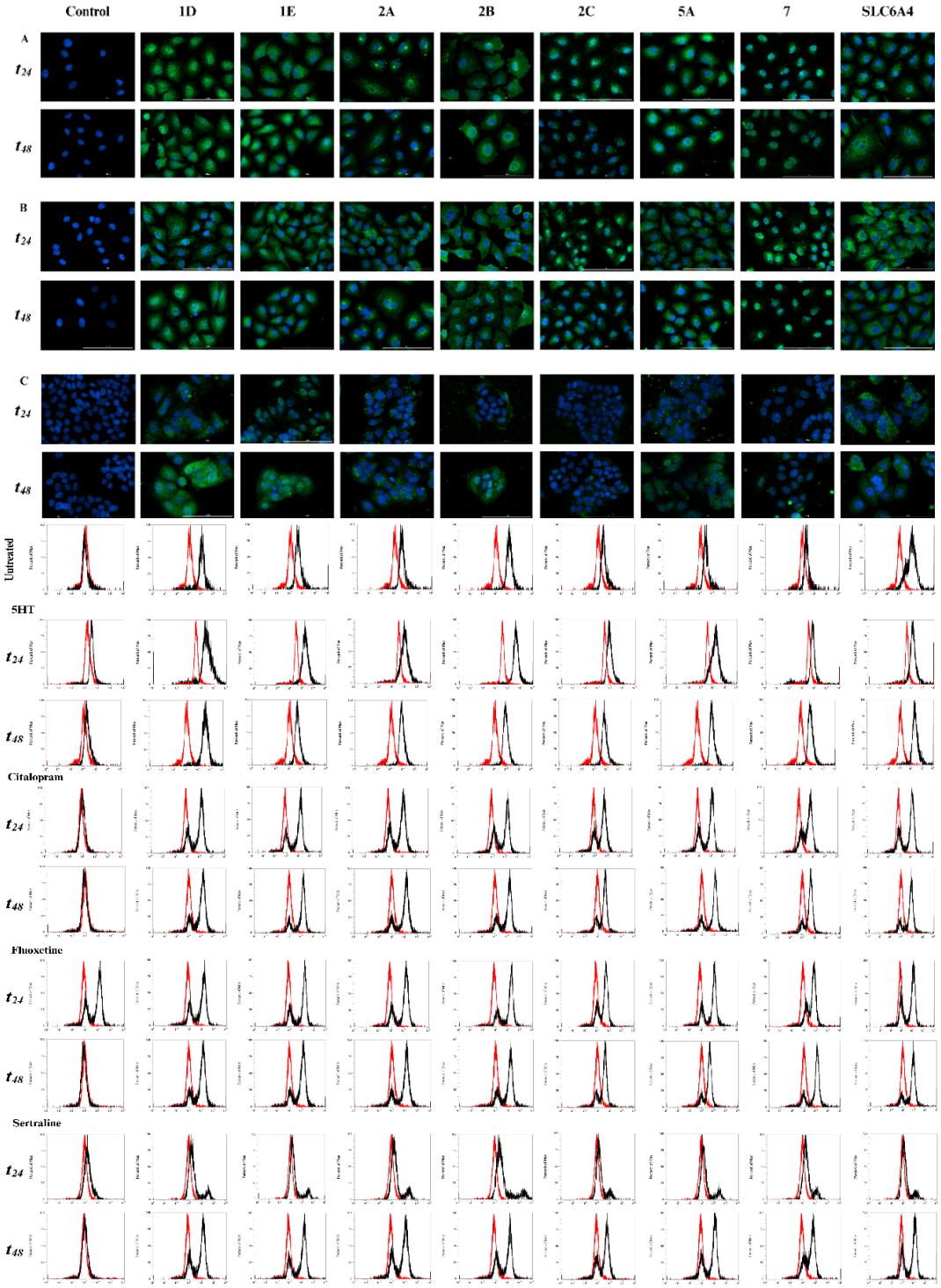

Figure 17 The Combined Effects of 5HT & SSRIs on 5HTR Expression in MDA-MB-231

The combined expression profile for serotonin receptors 5HTR$_{1B}$, 5HTR$_{1D}$, 5HTR$_{1E}$, 5HTR$_{2A}$, 5HTR$_{2B}$, 5HTR$_{2C}$, 5HTR$_{5A}$, and 5HTR$_{7}$, as well as the serotonin transporter (SLC6A4), was characterized using fluorescent microscopy (top) and quantified using flow cytometry (bottom) following 24 and 48 hours of 5HT and SSRI treatments. In the flow cytometry data, two peaks are represented following each treatment: the red peak on the left indicates control cells not incubated with a primary antibody, while the black peak represents cells incubated with a serotonin receptor antibody.

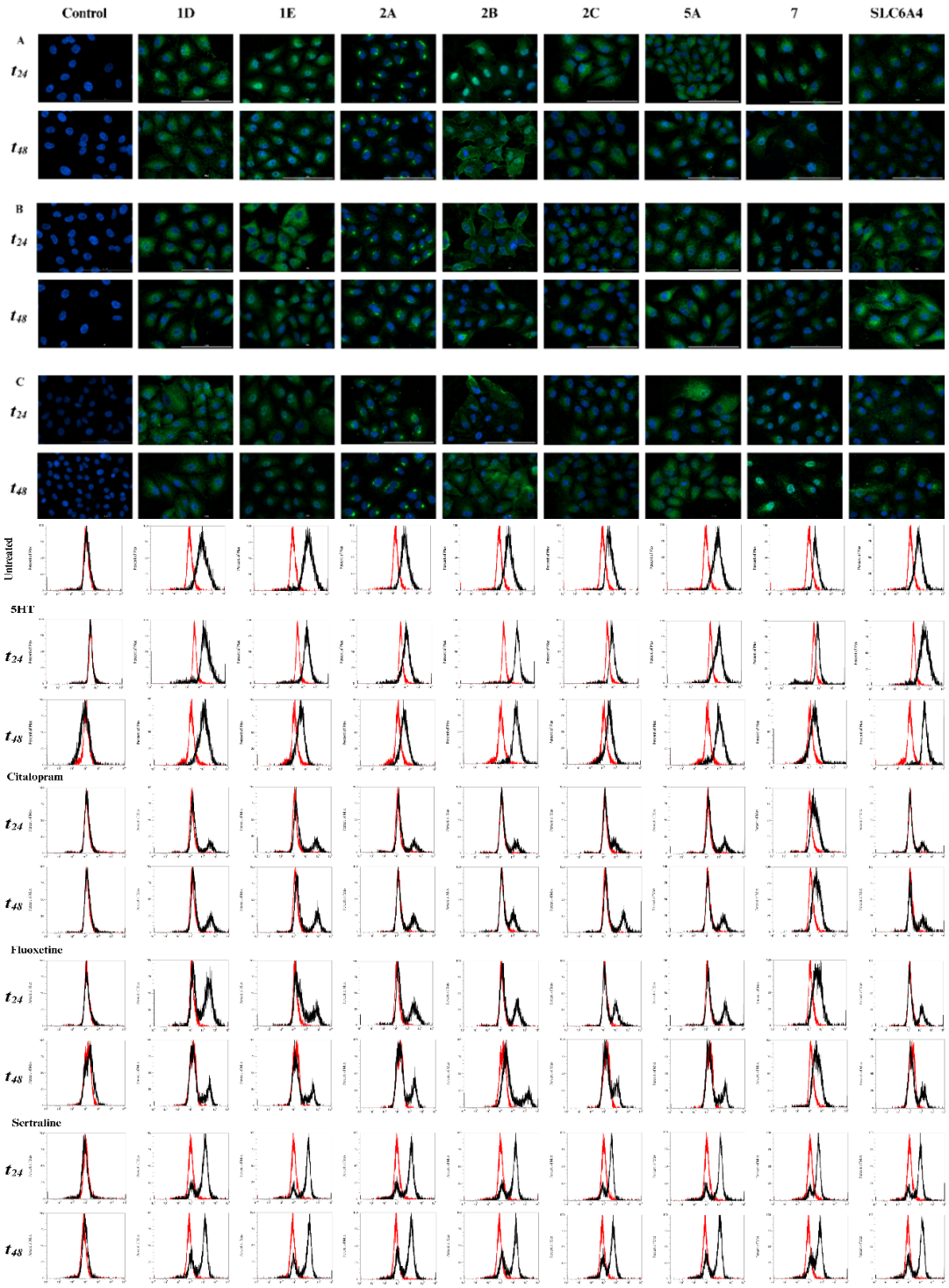

Figure 18 The Combined Effects of 5HT & SSRIs on 5HTR Expression in SK-N-AS

The combined expression profile for serotonin receptors $5HTR_{1B}$, $5HTR_{1D}$, $5HTR_{1E}$, $5HTR_{2A}$, $5HTR_{2B}$, $5HTR_{2C}$, $5HTR_{5A}$, and $5HTR_{7}$, as well as the serotonin transporter (SLC6A4), was characterized using fluorescent microscopy (top) and quantified using flow cytometry (bottom) following 24 and 48 hours of 5HT and SSRI treatments. In the flow cytometry data, two peaks are represented following each treatment: the red peak on the left indicates control cells not incubated with a primary antibody, while the black peak represents cells incubated with a serotonin receptor antibody.

			1D	1E	2A	2B	2C	5A	7	SLC6A4
A549	Cit	24 hrs	17.24±4.15	18.06±3.61	13.02±3.74	15.48±10.97	16.95±6.04	16.66±6.6	32.01±6.02	12.08±7.6
		48 hrs	25.3±5.33	26.2±8.25	24.84±5.81	24.38±9.24	23.19±4.04	18.87±7.52	47.42±10.85	18.87±3.5
	Flux	24 hrs	57.81±3.08	47.02±3.76	30.1±9.57	28.03±7.95	27.64±1.09	29.73±2.43	58.47±9.77	25.33±10.5
		48 hrs	22.22±8.83	21.06±7.58	26.67±4.4	33.03±4.96	26.53±7.58	22.05±1.74	36.25±4.49	18.64±5.6
	Sert	24 hrs	77.14±1.58	83.85±2.36	79.41±2.71	79.64±8.18	87.66±4.95	85.17±9.02	83.85±10.93	87.94±2.6
		48 hrs	74.35±10.79	75.91±3.22	79.01±1.1	77.74±10.14	76.92±8.73	73.41±8.93	75.71±5.41	72.86±4.5
Hs 578T	Cit	24 hrs	14.77±1.44	23.66±1.2	51.61±4.3	9.09±8.61	14.28±2.1	23.07±6.05	36.17±6.01	6.25±4.14
		48 hrs	16.66±10.69	21.05±10.32	48.27±6.81	55.88±5.23	9.09±3.5	33.33±2.03	34.78±4.8	34.78±4.0
	Flux	24 hrs	14.28±1.94	18.91±3.51	21.05±3.67	55.22±8.62	11.76±9.27	25±10.08	36.17±1.46	11.76±8.7
		48 hrs	16.66±6.29	14.28±1.42	16.66±7.93	11.76±6.78	18.91±3.09	14.28±4.12	43.39±4.72	9.09±3.05
	Sert	24 hrs	48.71±8.78	50.65±7.97	51.53±5.65	51.45±9.71	61.53±8.8	51.21±10.32	47.36±2.22	48.27±8.2
		48 hrs	47.82±4.73	49.57±4.94	50.16±4.91	67.84±3.88	43.39±8.45	51.45±1.57	49.06±8.22	39.75±2.4
MCF7	Cit	24 hrs	41.49±7.99	46.19±8.94	43.77±7.94	58.79±7.1	38.82±5.65	47.69±2.82	39.71±10.01	46.19±10.7
		48 hrs	41.19±1.87	43.08±6.61	42.34±6.96	54.78±6.07	35.51±1.76	46.68±3.77	40.7±4.3	44.42±8.8
	Flux	24 hrs	53.44±10.27	48.31±1.51	47.51±10.1	64.08±6.03	33.46±9.34	49.01±4.23	41.82±8.91	45.74±4.9
		48 hrs	53.03±9.81	54.51±1.32	56.33±1.95	62.03±1.52	38.08±7.62	52.4±7.03	41.83±10.42	48.45±3.5
	Sert	24 hrs	26.5±2.95	20.53±6.17	25.39±9.23	30.12±8.7	23.48±2.9	22.38±7.37	25.63±5.26	21.19±10.4
		48 hrs	46.11±2.26	46.11±4.95	42.18±6.09	62.79±9.76	38.21±7.65	46.8±7.06	47.14±7.18	40.32±7.7
MDA-MB-231	Cit	24 hrs	77.27±4.89	71.53±10.65	74.1±10.59	76.45±4.25	76.65±8.13	79.39±1	77.27±3.03	82.33±6.9
		48 hrs	82.33±7.41	84.61±9.37	72.75±7.98	82.08±2.76	83.08±3.49	83.58±4.97	80.4±3.44	86.75±10.1
	Flux	24 hrs	80.6±6.27	77.39±9.33	81.51±3.69	82.43±7.88	78.43±10.8	81.74±10.98	82.9±8.12	73.48±5.2
		48 hrs	74.71±2.74	74.07±8.65	78.31±10.61	71.23±4.35	83.87±6.16	86.95±9.11	87.54±7.71	83.87±5.0
	Sert	24 hrs	21.59±1.6	18.9±10.5	17.77±8.26	37.06±6.82	11.61±9.27	20.27±9.46	36.72±5.01	59.72±2.7
		48 hrs	69.79±4.13	75.28±5.84	68.71±8.19	69.97±8.65	74.63±2.54	69.79±2.95	76.79±4.87	69.97±8.8
SKNAS	Cit	24 hrs	17.24±6.63	18.06±3.94	13.02±8.1	15.48±4.93	16.95±9.19	16.66±5.95	32.01±6.56	12.08±6.0
		48 hrs	25.3±9.01	26.2±10.3	24.84±7.13	24.38±9.13	23.19±9.16	18.87±1.58	47.42±4.13	18.87±10.6
	Flux	24 hrs	57.81±8.16	47.02±4.65	30.1±2.32	28.03±9.35	27.64±5.79	29.73±3.04	58.47±9.12	25.22±2.3
		48 hrs	22.22±10.4	21.06±1.01	26.67±9.01	33.03±9.55	26.53±7.07	22.05±10.71	36.25±1.94	18.64±8.7

Table 5 The Effects SSRIs on 5HTR Expression

The inhibitory effects of SSRIs on serotonin receptor and the serotonin transporter as determined by flow cytometry as determined by an

4 Discussion

4.1 Serotonin Receptor Expression

Establishing a baseline expression of serotonin receptors was critical for understanding the role of serotonin receptors in cancer development and progression. In this study, we assessed the expression of serotonin receptors ($5HTR_{1B}$, $5HTR_{1D}$, $5HTR_{1E}$, $5HTR_{2A}$, $5HTR_{2B}$, $5HTR_{2C}$, $5HTR_{5A}$, and $5HTR_{7}$) and the serotonin transporter (SLC6A4) in several cell lines (A549, Hs 578T, MCF7, MDA-MB-231, and SK-N-AS) using immunofluorescence imaging and flow cytometry. This was also the first time that such a large panel of serotonin receptors were identified in several different cancer types. Immunofluorescence imaging revealed that all receptors, except for $5HTR_{1B}$, show a consistent and homogenous level of expression across all cell lines, which was also confirmed through flow cytometry. It should be noted however, that the lack of $5HTR_{1B}$ expression could have been due to the specificity of the antibody since it has been shown to be expressed in several cancer types (Table 1). Regardless, this baseline expression profile confirms many of the findings in the previous studies as well as the expression of almost half of known serotonin receptors in several cancer types (Table 1). It could also be extrapolated that each of the cell lines investigated here may express all the serotonin receptors, and through their collective functionalities, affect development and progression.

Upon treatment with 1 μM serotonin, a notable transient upregulation of receptor expression was observed. Immunofluorescence imaging showed a similar pattern for each 5HT receptor as compared to the baseline profile, with areas of serotonin receptors centered around the nucleus, as seen for $5HTR_{2A}$. Similarly, the flow cytometry results revealed a rightward shift in receptor expression after 24 hours. After 48 hours, the expression levels

returned to baseline, suggesting a transient upregulation in response to serotonin. This transient nature of receptor upregulation is consistent with the literature suggesting that serotonin can modulate receptor expression levels temporarily.[72,74] This mechanism could be compensatory to enhanced serotonergic signaling in response to increased extracellular serotonin levels.

When cell lines were treated with 10 μM of citalopram, fluoxetine, or sertraline for 24 and 48 hours, significant changes in receptor expression pattern and levels were noted. Immunofluorescence microscopy indicated fewer and more localized regions of receptor presence compared to the homogenous distribution seen with serotonin treatment, suggesting that SSRIs might induce receptor internalization or degradation.[61,63,204] However, our data suggests that on average, the $5HTR_7$ receptor generally exhibited the highest levels of serotonin receptor expression and the $5HTR_2$ family of serotonin receptors exhibited the lowest levels of expression in each cell line. suggesting that certain serotonergic receptors or families respond differently to changes in extracellular serotonin. This seems to be further supported by the fact that $5HTR_7$ and the $5HTR_2$ family of serotonin receptors are often implicated in the development and progression of cancer, perhaps due to their sensitivity to serotonin.[11,74,130,147] Traditionally, the administration of SSRIs is meant to block the reuptake of serotonin to increase the concentration of extracellular serotonin available to interact with serotonin receptors. Yet, SSRI treatments lead to a decrease in serotonin receptor expression. This may be due to the counter intuitive nature of serotonin signaling in cancer, where serotonin has been shown to take part in autocrine loops affecting growth in several cancer types.[74,142,162,205]

Flow cytometry data revealed that citalopram treatment generally led to decreases in serotonin receptor expression levels, while sertraline treatment increased the expression levels. MDA-MB-231 exhibited the highest levels of serotonin receptor expression levels following treatments with citalopram and fluoxetine, suggesting possible resistance mechanisms or differences in receptor regulation pathways. This could also be due to the inherent characteristics of the MDA-MB-231 cells, known for their aggressive and metastatic nature, which might affect their responsiveness to SSRIs.[206] This is most likely attributed to the different binding specificities of each SSRI. Yet this does not explain why the $5HTR_{5A}$ receptor and the $5HTR_{1}$ family exhibited such different expression patterns in each cell line, while not exhibiting the changes in expression location as seen in $5HTR_{2A.}$ Regardless, the effects seen in the other cell lines align with studies indicating SSRIs can lead to receptor desensitization and downregulation likely through binding to the serotonin transporter and decreasing serotonin reuptake, leading to receptor desensitization.[110,195,207,208]

Previously, reports on serotonin receptor expression and expression levels in different cancer types were scattered and inconsistent. The results of this study showed that several serotonin receptors were present in each cell line, and perhaps hinting that all the serotonin receptors are present as well. This study provides a much needed foundation to better understand the role of serotonin receptors in cancer development and progression. Moving forward, studies are needed that focus on these receptors and their mechanism of action in cancer. In addition, this study showed that serotonin receptor expression was affected by

SSRI treatments which could have meaningful clinical implications for patients diagnosed with cancer who are taking SSRIs. Specifically, how those SSRIs and their secondary metabolites may interact with cancer and affect their prognosis.

4.2 The Effects of Serotonin and SSRIs on Cell Growth and Metastasis

The combined treatment of 5HT with SSRIs revealed complex interactions, highlighting the necessity to consider the dual role of serotonin as both a neurotransmitter and a modulator of cellular proliferation. Generally, there was no significant effect on cell growth regardless of treatment. However, the transient response observed suggests a fast-acting nature of serotonin or SSRIs and together with the fact that treatments with SSRIs and SSRIs+5HT increased cell growth more than treatments with just serotonin warrant further investigation. This is especially true since serotonin has been associated with increased cell growth as well as a marker for higher tumor grade and metastatic cancers.[43,72,74,209,210]

Cancer metastasis is intricately linked to cell growth, as motile cells can readily access distant tissues and establish secondary tumors.[4,5] Serotonin has recently emerged as a potential player in cancer development and progression, interacting with various receptors influencing their migratory behavior.[6] SSRIs, through their action of elevating extracellular 5HT levels, have also been implicated in the development of metastasis. While treatments with serotonin did not significantly impact the migratory velocity in this study, treatments with SSRIs and SSRIs+5HT did show varying levels of effect on migration, generally decreasing migratory velocity. The most significant effect was observed upon sertraline and sertraline+5HT treatments. Throughout this study, sertraline

has consistently shown the most significant effects. Again, hinting to a better or more efficient binding affinity. It should also be noted that according to the literature, serotonin has a mitogenic and pro-metastatic effect.[4,43,72,74,130] Therefore, the fact that treatments with sertraline and sertraline+5HT decreased relative migratory velocity in the lung cancer cell line A549 and the triple-negative cell lines Hs 578T and MDA-MB-231 after 12 and 24 hours, might be contrary to the greater body of work done so far, since theoretically, SSRIs increase the amount of serotonin in the extracellular space, which then in turn should lead to increased serotonin receptor activity and a greater metastatic effect. However, our results might be indicative of an increase of metastatic ability or increased mobility in other parts of the metastatic cascade, warranting further and more specific experimentation

4.3 Strengths, Weaknesses and Future Directions

The strength of this study lies in its comprehensive approach, utilizing both immunofluorescence imaging and flow cytometry to assess receptor expression. This dual-method approach provides robust validation of findings, ensuring that observed changes in receptor expression are reliable and not artifacts of a single detection method. Immunofluorescence allows visualization of receptor localization, while flow cytometry provides quantitative analysis of expression levels. Moreover, this is the first time that such a large number of serotonin receptors and the serotonin transporter were shown to be expressed in several cancer types.

However, there are limitations. Firstly, the use of only three SSRIs at a fixed concentration may not capture the full spectrum of their effects on serotonin receptor expression. Different SSRIs have varied affinities for serotonin receptors, and their effects

can be dose-dependent. Future studies should explore a range of concentrations and additional SSRIs for a more comprehensive understanding, since this study mainly served to determine which serotonin receptors were expressed in different cancer types. Secondly, the transient nature of observed effects highlights the need to observe the effects of serotonin and SSRIs in shorter timepoints from hours to perhaps even minutes. This is especially true since the half-life of SSRIs are relatively short and the effects of the SSRIs and serotonin might be occurring shortly after administration.[96,115,212] On the other hand, studies involving the continuous administration of SSRIs and serotonin would prove useful as well for that same reason. Furthermore, while this study focused on receptor expression, it did not address functional changes in receptor activity. Future studies should include assays to measure receptor signaling activity. Finally, while informative, animal models should be utilized to better understand the effects of serotonin and SSRIs *in viv*o.

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