Department of Molecular Pharmacology and Neuroscience

Characterizing the Transport Dynamics of the False Fluorescent Neurotransmitter FFN246 in a SERT-Expressing Cellular Model

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Introduction

The neurobiological mechanisms of psychiatric disorders represent a central focus of contemporary neuroscience research, with particular emphasis on monoaminergic neurotransmission as a primary mediator of behavioral and affective dysregulation. Among the monoamines, dopamine, norepinephrine, and serotonin alterations in signaling have been consistently implicated in the pathophysiology of a broad spectrum of neuropsychiatric conditions (Jesulola et al., 2018). Serotonin (5-hydroxytryptamine; 5-HT) is a particular mechanistic interest, functioning as a critical modulatory neurotransmitter in the regulation of mood, sleep, appetite, cognitive processing, and nociception (Jesulola et al., 2018). Biosynthesized primarily within the raphe nuclei of the brainstem, serotonergic projections innervate extensive regions of the central nervous system via ascending and descending neural pathways, enabling its broad influence over both limbic and cortical function (Krystal, 2001).

Dysregulation of serotonergic neurotransmission has been extensively implicated in the pathophysiology of numerous psychiatric disorders, with major depressive disorder (MDD) representing one of the most clinically significant manifestations (Nemeroff, 2009). The monoamine hypothesis provides evidence that deficiencies in serotonergic tone at synaptic terminals contribute to the onset of depressive symptoms (Jesulola et al., 2018). Pharmacological support for this framework derives from the mechanism of action of selective serotonin reuptake inhibitors (SSRIs), which potentiate serotonergic neurotransmission by competitively inhibiting the serotonin transporter (SERT), thereby attenuating synaptic reuptake and prolonging receptor activation (Vergne, 2006). Nevertheless, the clinical management of MDD remains considerably complex, as therapeutic response is highly variable across patient populations. Differences in pharmacogenomic profiles, including polymorphisms in genes encoding drug-metabolizing enzymes and synaptic targets, require iterative pharmacological adjustment, often requiring multiple treatment trials before achieving adequate symptom remission (Nemeroff, 2009).

Beyond MDD, dysregulation of serotonergic signaling has been broadly implicated in the pathophysiology of a diverse array of neuropsychiatric and somatic conditions, including obsessive-compulsive disorder (OCD), anxiety disorders, eating disorders, and chronic pain syndrome (Frick, 2015; Griebel, 1995). Consequently, identifying the molecular mechanisms governing serotonin biosynthesis, synaptic release, transporter mediated reuptake, and postsynaptic receptor interactions remain a critical area for the development of more targeted and efficacious pharmacotherapeutic strategies.

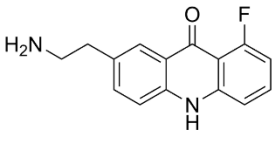
Investigators at Columbia University have developed a class of innovative imaging tools termed false fluorescent neurotransmitters (FFNs) which function as fluorescent substrates that enable visualization and quantitative assessment of neurotransmitter uptake dynamics at individual synaptic terminals, both in vitro and ex vivo (Henke, 2017). These methodological advancements have provided insights into the kinetics of serotonin transport and hold considerable promise for furthering mechanistic characterization of serotonin transporter (SERT) and vesicular monoamine transporter-2

(VMAT-2), two integral membrane proteins central to the regulation of serotonergic neurotransmission (Wang, 2024).

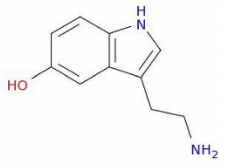
Despite these advances, significant gaps remain in the characterization of FFN246's transport properties and the boundary conditions governing its utility as a serotonergic imaging tool. The present study sought to address this limitation by examining FFN246 transport kinetics in human embryonic kidney (HEK293) cells transfected with SERT, providing a well-controlled system for isolating transporter-specific uptake and efflux dynamics at the transporter level. Identification of a compound capable of replicating endogenous 5-HT transport properties would establish a pharmacological model for investigating the mechanistic basis of SERT-mediated efflux, a process of relevance to the development of novel therapeutic agents targeting monoaminergic neurotransmission.

We hypothesize that if FFN246 demonstrates suitability as a functional substrate for SERT-mediated transport, its validated use as a fluorescent proxy for 5-HT would enable the design of a systematic, high-throughput screening tool to evaluate efflux-inducing properties of multiple drug candidates in HEK293 cells transfected with SERT. The development of such a screening tool would substantially enhance the efficiency of early-stage drug discovery by enabling high volume assessment of candidate compounds prior to confirmation by orthogonal methodologies. Establishing FFN246 as a reliable reporter of SERT-mediated efflux would represent a meaningful methodological contribution, bridging the gap between preliminary fluorescent-based screening and mechanistic validation, ultimately accelerating the identification of novel therapies capable of modulating serotonergic neurotransmission.

The primary aim of the present study was to characterize FFN246 transport kinetics through fluorometric assessment utilizing the BioTek Cytation plate reader, with optimization of the experimental parameters to ensure assay reproducibility and sensitivity. A secondary objective was to evaluate the broader translational potential of FFN246 as a novel methodological tool for advancing mechanist serotonin research, specifically through the establishment of a validated fluorescence-based protocol capable of detecting SERT-mediated uptake and efflux dynamics that have not been previously described in the literature.



FFN246
-Molecular Weight: 256.27 g/mol

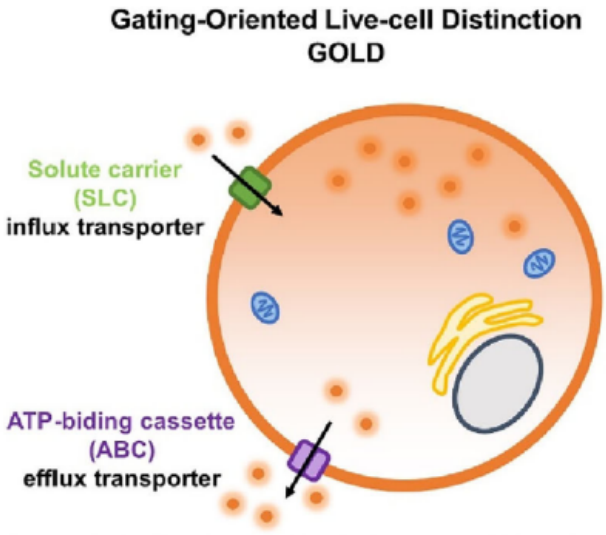


5-HT
-Molecular Weight: 176.21 g/mol

Experimental Methods

FFN-246 Preparation

The FFN246 solution was prepared in uptake buffer to facilitate simultaneous introduction of the fluorescent ligand and buffer. A 5 mM stock solution was used to achieve a final concentration of 5 µM, determined as optimal for loading the fluorescent ligand. Approximately 10 µL of FFN246 stock solution was added to 4990 µL of uptake buffer and vortexed thoroughly. The FFN246 solution was subsequently placed on a plate warmer set at 37 degrees Celsius until the start of experiment.



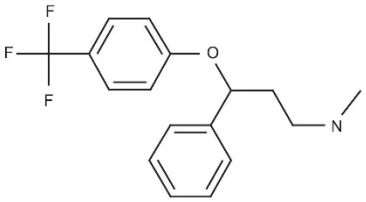
Uptake Buffer Preparation

To prepare the Uptake Buffer, first measure out 0.1 g (100 mg) of Glucose and 10 mg of Ascorbic Acid into separate 1 mL centrifuge tubes. In a graduated cylinder, measure 5 mL of 20X Buffer, then add MilliQ water until the total volume reaches 95 mL. Transfer the solution from the graduated cylinder into the centrifuge tubes containing the Glucose and Ascorbic Acid to solubilize them. Vortex the centrifuge tubes to ensure thorough mixing, then use a pipette to transfer the contents back into the graduated cylinder. Next, add 125 µL of Magnesium Sulfate and 62.5 µL of Calcium Chloride to the graduated cylinder and gently mix.

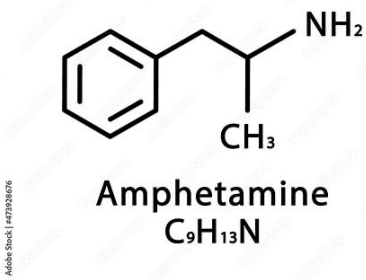
Transfer the contents of the graduated cylinder into an appropriately sized beaker containing a magnetic stir bar and place on a stir plate. Using a calibrated pH meter, measure the pH of the solution and adjust to 7.4 by dropwise addition of 1M NAOH or 1M HCL as required. Once the desired pH is reached, transfer the contents from the beaker back into the graduated cylinder and fill it up to 100 mL with MilliQ water. The Uptake Buffer is now prepared. Transfer it back into the beaker and keep it on a hot plate or water bath set to 37°C until needed for the experiment.

Drug Candidate Preparation

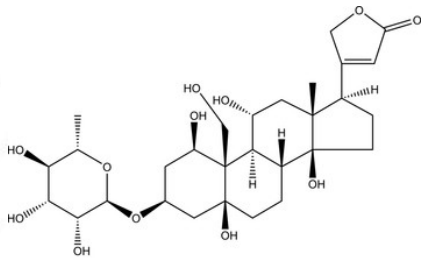
Drug candidates were prepared using an identical protocol for FFN246, facilitating the simultaneous addition of uptake buffer and pharmacological agents to HEK293 cells. A 5mL stock solution of each compound in uptake buffer served as the basis for subsequent dilution series. Working concentrations were achieved primarily through serial dilution, ensuring consistent and reproducible dosing across experimental replicates. The pharmacological agents investigated in the present study comprised of fluoxetine, amphetamine, and ouabain.



Fluoxetine



Amphetamine
$C_9H_{13}N$



Ouabain ($C_{29}H_{44}O_{12}$)

Uptake Assay

Begin by obtaining a plate with HEK293 cells transfected with SERT. To each well, add 100 µL of uptake buffer. For the testing groups, also add 5 µM of FFN246 to the wells. Allow the plate to incubate for 20 minutes. After the incubation period, use a vacuum to remove the uptake buffer from the wells. Wash the wells three times by adding 200 µL of 1X PBS and removing it with the vacuum.

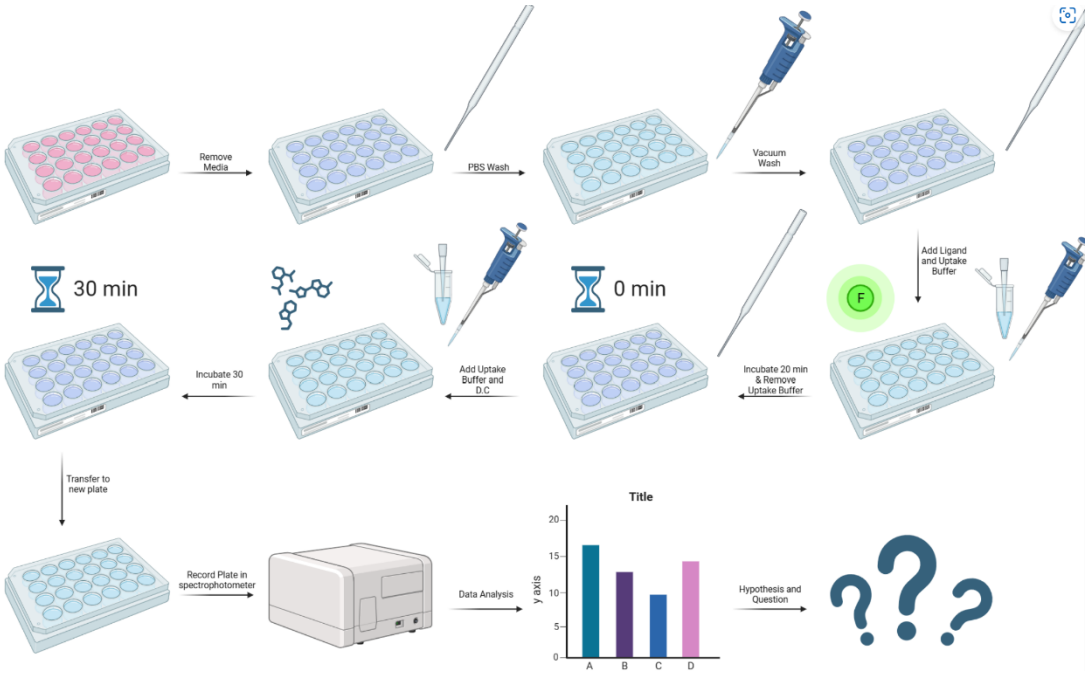
Upon completion of the washing step, the 96-well plate was transferred to the BioTek Cytation plate reader and instrumental parameters were configure prior to data acquisition. For FFN246 detection, excitation and emission wavelengths were set to 388 nm and 434 nm, respectively. For yellow fluorescent protein (YFP), excitation and emission wavelengths were set to 490 nm and 530 nm respectively. Following the confirmation of all parameters, fluorescent measuring was performed.

Efflux Assay

Begin by obtaining a plate with HEK293 cells transfected with SERT and add 100 µL of uptake buffer containing the fluorescent ligand to the HEK cells in media in a well plate. Note that the total volume will be 200 µL (100 µL media + 100 µL uptake buffer). Allow the plate to incubate for 20 minutes. After incubation, remove the uptake solution from each well using a vacuum.

Next, perform a 3X wash by adding 200 µL of PBS to each well, then removing it with the vacuum. Add 200 µL of efflux buffer containing amphetamines to each well and incubate again for 10 minutes. After the incubation period, carefully collect 100 µL of the efflux solution from each well and transfer it to a new 96-well plate.

Perform another 3X wash on the original plate by adding 200 µL of PBS, then removing it with the vacuum. You should now have two 96-well plates - the original plate with cells, and the new plate containing the collected efflux solutions. Place both plates into the plate reader and set the running parameters. For FFN246, set excitation to 388 nm and emission to 434 nm. For YFP, set excitation to 490 nm and emission to 530 nm.



Results

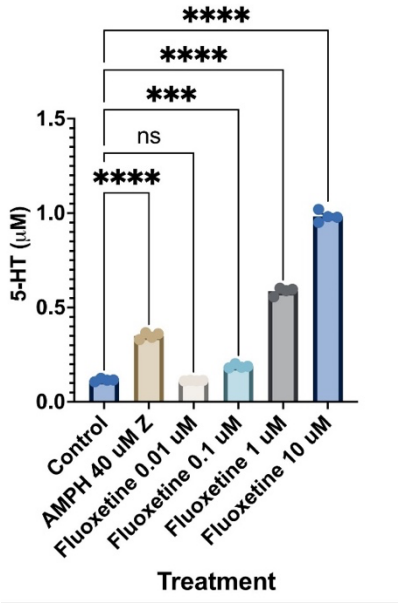


Figure 1 – SERT-Mediated Efflux in HEK293 Cells Expressing SERT: Concentration Dependent Effects of Fluoxetine. Fluoxetine induced significant 5-HT Efflux at concentrations of 1 µM and 10 µM, with efflux measurement exceeding that of the 40 µM positive control (amphetamine), suggesting concentration-dependent efflux profile (Study Prompting Investigation into FFN246).

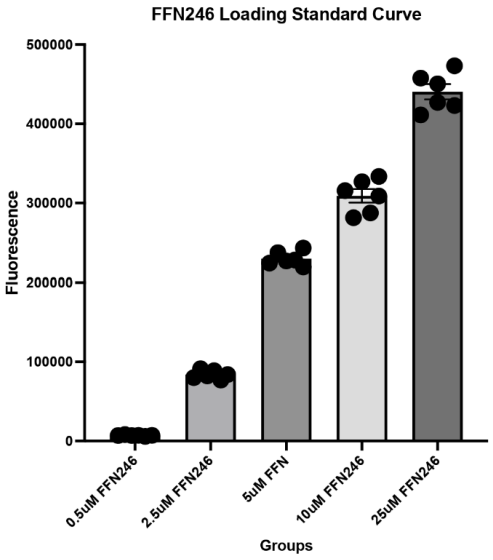


Figure 2 – FFN246 uptake was assessed HEK293 cells expressing SERT across a concentration range of 0.5 µM, 2.5 µM, 5 µM, 10 µM, and 25 µM. Fluorescence intensity is reported in arbitrary units (A.U.), reflecting relative FFN246 accumulation within SERT-expressing cells as a function of substrate concentration

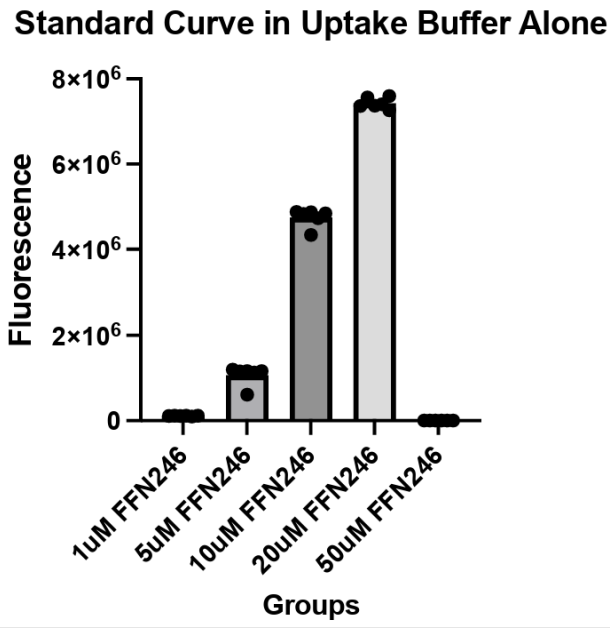


Figure 3 – A standard curve of FFN246 was generated in uptake buffer across a concentration range of 1 µM, 5 µM, 10 µM, and 20 µM. Fluorescence intensity reported in arbitrary units (A.U.) as a function of FFN246 concentration. Note that the 50 µM condition exceeded the dynamic range of the BioTek Cytation plate reader, resulting in a signal saturation.

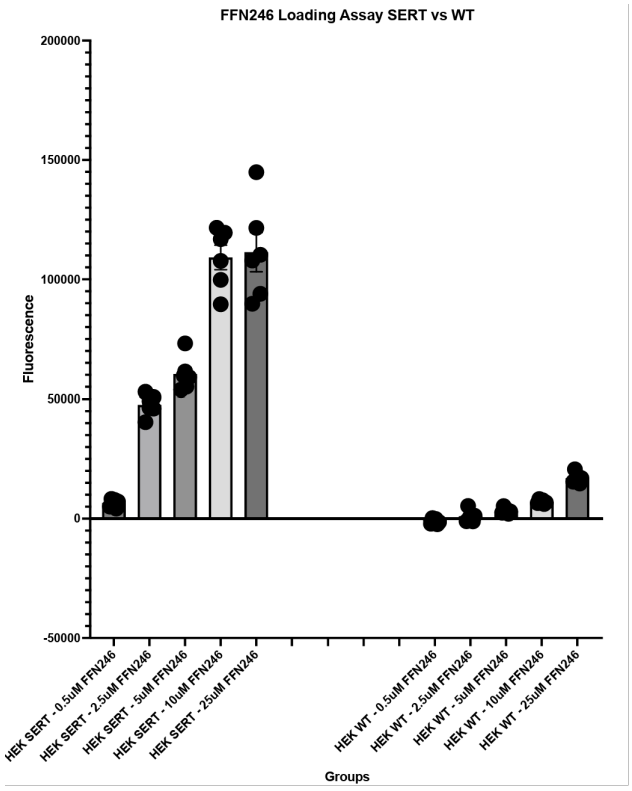


Figure 4 – Concentration-dependent uptake of FFN246 was assessed in HEK293 cells expressing SERT (HEK-SERT) compared to wildtype HEK293 cells (HEK-WT) across a concentration range of 0.5 µM, 2.5 µM, 5 µM, 10 µM, and 25 µM. Fluorescence intensity is reported in arbitrary units (A.U.), reflecting relative FFN246 accumulation as a function of substrate concentration. HEK-SERT and HEK-WT conditions are presented in the left and right panels, respectively.

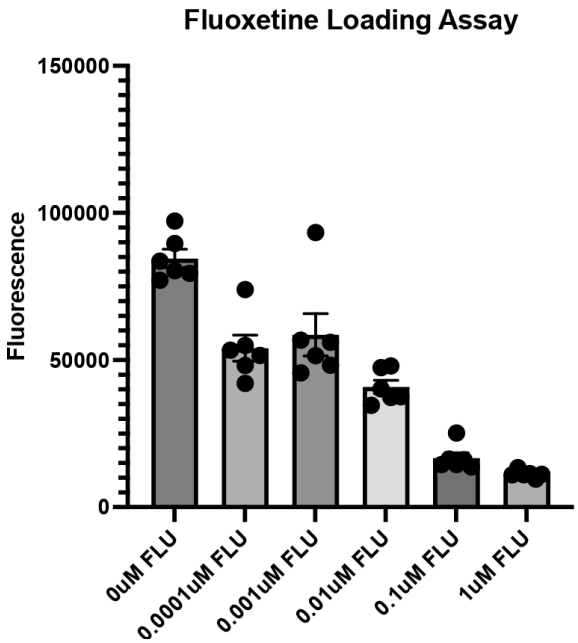


Figure 5 – Uptake inhibition of 5 µM FFN246 was assessed in HEK293 cells expressing SERT in the presence of fluoxetine across a concentration range of 0.0001 µM, 0.001 µM, 0.01 µM, 0.1 µM, and 1 µM. Fluorescence intensity is reported in arbitrary units (A.U.), reflecting relative FFN246 accumulation as a function of increasing fluoxetine concentration.

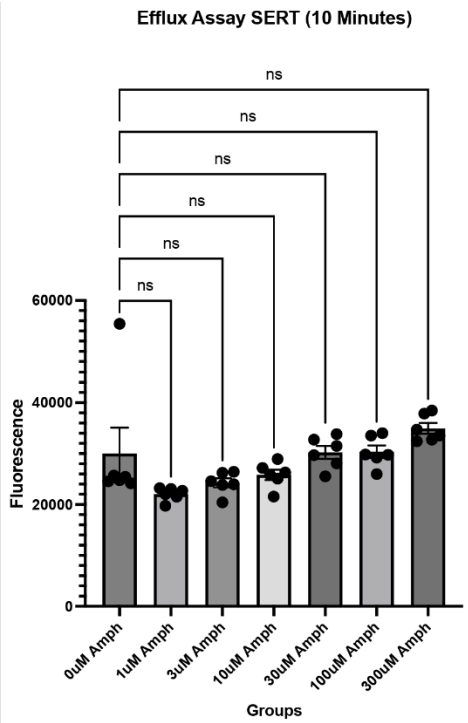


Figure 6 – SERT-mediated efflux of 5 µM FFN246 was assessed in HEK293 cells expressing SERT by quantification of supernatant fluorescence following treatment with amphetamine across a concentration range of 1 µM, 3 µM, 10 µM,30 µM, 100 µM, and 300 µM. Fluorescence intensity is reported in arbitrary units (A.U.).

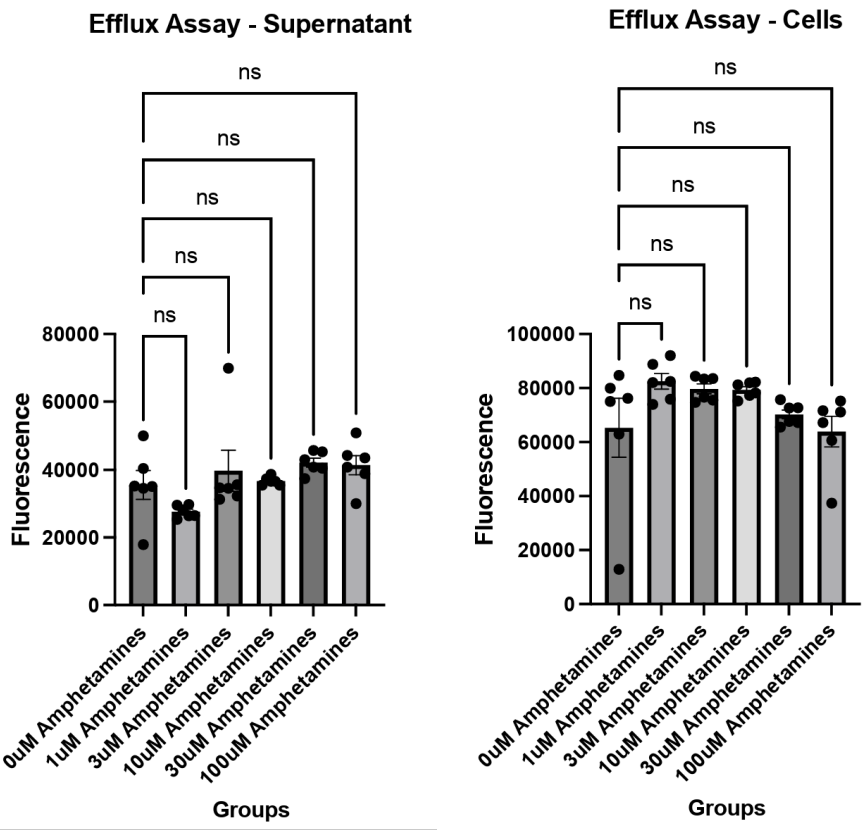


Figure 7 – SERT-mediated efflux of 5 µM FFN246 was assessed in HEK293 cells expressing SERT following treatment with amphetamine across a concentration range of 1 µM, 3 µM, 10 µM, 30 µM, and 100 µ. Fluorescence intensity reported in arbitrary units (A.U.). The left panel depicts relative FFN246 accumulation in the supernatant fraction, reflecting efflux into the extracellular compartment as a function of increasing amphetamine concentration. Right panel depicts residual intracellular FFN246 fluorescence measure in cells following supernatant collection and three successive wash steps, providing an index of retained substrate after efflux.

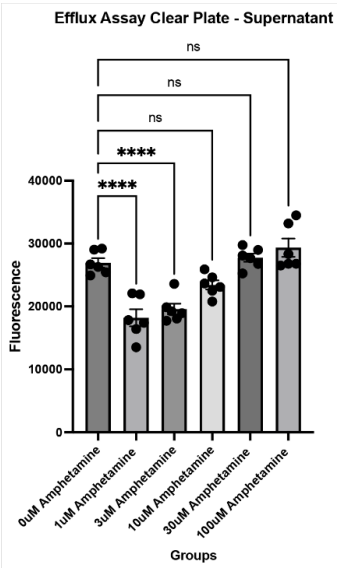


Figure 8 – Final SERT-Mediated efflux assay of 5 µM was assessed in HEK293 cells expressing SERT following treatment with amphetamine across a concentration range of 1 µM, 3 µM, 10 µM, 30 µM, and 100 µM. Fluorescence intensity is reported in arbitrary units (A.U.), reflecting relative FFN246 efflux into the extracellular compartment as a function of increasing amphetamine concentration.

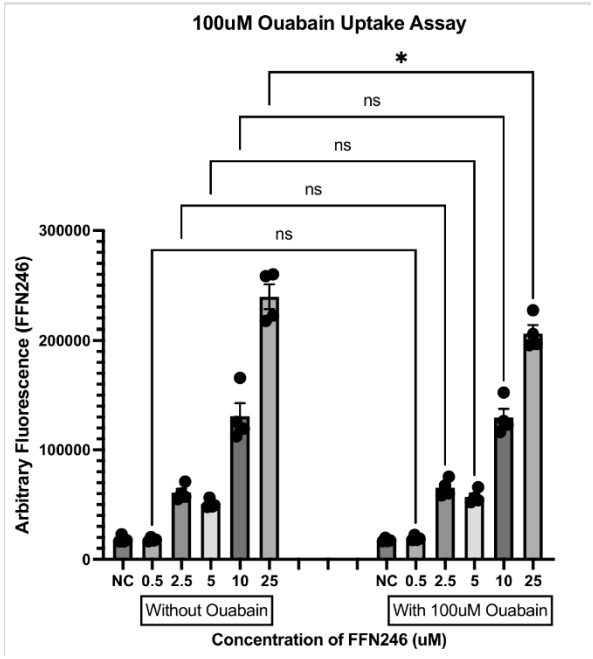


Figure 9 – Concentration-dependent uptake of 0.5 µM, 2.5 µM, 5 µM, 10 µM, and 25 µM FFN246 was assessed in HEK293 cells expressing SERT in the presence and absence of ouabain (100 µM), a selective NA+/K+-ATPase inhibitor. Fluorescence intensity is reported in arbitrary units (A.U.), reflecting relative FFN246 accumulation as a function of substrate concentration. The left panel depicts FFN246 uptake in untreated HEK293-SERT cells, while the right panel depicts FFN246 uptake following ouabain treatment, enabling direct comparison of transporter-dependent dynamics under conditions of NA+/K+ ATPase inhibition.

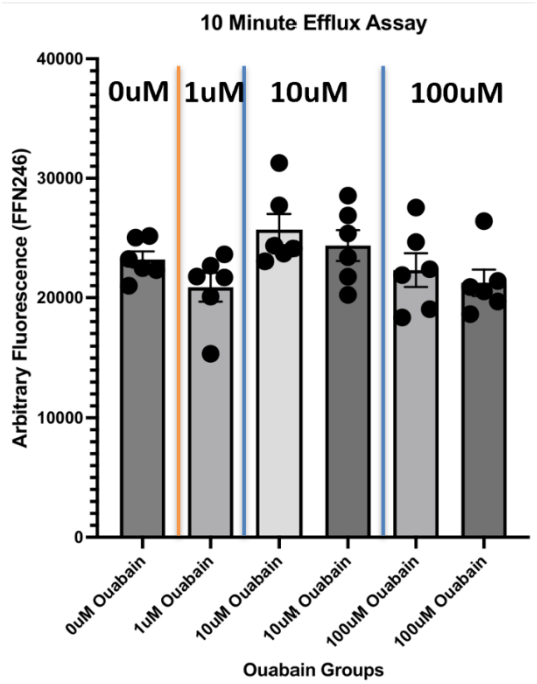


Figure 10 – SERT-mediated efflux of 5 µM FFN246 was assessed in HEK293 cells expressing SERT following treatment ouabain across a concentration range of 1 µM, 10 µM, and 100 µM. Fluorescence intensity is reported in arbitrary units (A.U.), reflecting relative FFN246 efflux into the extracellular compartment as a function of increasing ouabain concentration. Duplicate independent measurements were performed at 10 µM and 100 µM concentrations and are presented as separate data series to permit assessment of inter-replicate reproducibility.

Discussion

The first series of experiments aimed to establish the fundamental transport properties of FFN246 in a SERT-expressing cellular system. To accomplish this, a concentration-dependent uptake assay was performed in HEK293-SERT cells, revealing a clear positive relationship between FFN246 concentration and cellular fluorescence signal. This concluded that as more FFN246 was introduced, more was taken by the cells (Figure 2). This finding provided an initial indication that FFN246 behaves as a functional substrate of SERT, accumulating within cells in a manner consistent with active transporter-mediated uptake rather than passive diffusion. To further validate the instrument's capacity to detect FFN246 across the concentration range of interest, a standard curve was generated in uptake buffer alone, independent of any cellular system. This assessment revealed that at 50 μM, the fluorescence signal exceeded the upper detection limit of the BioTek Cytation plate reader, producing a signal saturation (Figure 3).

Based on the uptake and standard curve data, a working concentration of 5 μM was selected as optimal for FFN246 loading experiments (Figures 2 & 3). This concentration struck a practical balance between two competing requirements: it was sufficiently high to produce a robust and clearly detection range, ensuring accurate quantification of FFN246 in both the cellular and supernatant fractions. The ability to reliably measure FFN246 in the supernatant is important, as this fraction serves as a proxy for transporter mediated efflux, similar to serotonin release from neurons back into the synapse under certain physiological and pharmacological conditions. Together, these foundational experiments established 5 μM FFN246 as the standardized loading concentration for all subsequent assays, providing a reproducible framework for investigating serotonin transport dynamics using this novel fluorescent tool.

Having established optimal loading conditions, the next step was to determine whether FFN246 uptake was specifically mediated by SERT rather than entering cells through non-specific mechanisms. To address this, FFN246 uptake was compared between HEK293-SERT and HEK293-WT cells across the same concentration range used in previous experiments (0.5 μM, 2.5 μM, 5 μM, 10 μM, and 25 μM). HEK293-SERT cells displayed substantially greater FFN246 accumulation than their wildtype counterparts at all concentrations tested, demonstrating that uptake was driven primarily by SERT-mediated transport rather than passive cellular entry (Figure 4). This distinction is critical, as it confirms that FFN246 fluorescence in subsequent assays can be attributed predominately to SERT activity, providing confidence that the compound is functioning as intended as a fluorescent proxy for 5-HT transport.

Notably, however, HEK293-WT cells did exhibit a small but detectable degree of FFN246 uptake at the two highest concentrations tested, 10 μM and 25 μM, suggesting that SERT is not the sole route of cellular entry for FFN246 at elevated concentrations

(Figure 4). This observation points to possible involvement of additional transport mechanisms, such as ion channels or other membrane-associated protein transports, that may facilitate FFN246 influx independent of SERT. This is not entirely unexpected, it is well established in the fluorescent imaging literature that fluorescent compounds can access cells through multiple pathways, both for influx and efflux, particular at higher concentrations where non-specific membrane interactions become increasingly likely (Choi, 2019). While this non-specific uptake does not undermine the validity of FFN246 as a SERT substrate, it does underscore the importance of working within optimized concentration range, most critically at 5uM, where SERT-mediated transport dominates and non-specific signals remain negligible.

Taken together, these findings reinforce the central role of SERT in mediating FFN246 uptake while simultaneously drawing attention to the existence of alternative SERT-independent transport pathways in HEK293-WT cells (Figure 4). Rather than diminishing the utility of FFN246 as a serotonergic imaging tool, this complexity adds an interesting depth to its characterization, one that warrants further investigation. Understanding the nature of these alternative pathways, whether mediated by ion channels, non-selective membrane transporters, or concentration-driven passive mechanisms, could meaningfully broaden our understanding of FFN246 transport dynamics beyond its interaction with SERT alone. Such insights would not only refine the experimental conditions under which FFN246 can be most reliably employed but could also reveal previously unappreciated aspects of fluorescent neurotransmitter analog behavior that may have broader implications for the field of serotonin transport research.

To further investigate the specificity of SERT-mediated FFN246 uptake, a pharmacological inhibition approach was utilized with fluoxetine, a widely prescribed SSRI clinically indicated for the treatment of depression and anxiety disorders. By introducing fluoxetine both prior to and during FFN246 loading, SERT activity could be selectively blocked, allowing us to isolate the transporters specific contribution to FFN246 uptake. IF FFN246 entry into cells is genuinely dependent on SERT, then blocking the transporter with fluoxetine should produce a measurable, concentration-dependent reduction in cellular fluorescence. To test this, HEK293-SERT cells were treated with fluoxetine across a broad concentration range spanning four orders of magnitude, 0.0001 μM, 0.001 μM, 0.01 μM, 0.1 μM, and 1 μM, in the presence of a fixed 5 μM FFN246 concentration, enabling systematic evaluation of relationship between SERT inhibition and FFN246 uptake (Figure 5).

The results of the fluoxetine inhibition assay provided some of the most compelling evidence yet for the specificity of SERT-mediate FFN246 uptake. As fluoxetine concentrations increased across the tested range, a clear and progressive reduction in

cellular FFN246 fluorescence was observed, consistent with dose-dependent SERT inhibition (Figure 5). In practical terms, the more effectively fluoxetine blocked SERT, the less FFN246 was able to enter the cell, a relationship that directly mirrors the way serotonin uptake is suppressed in patients treated with SSRIs. This concentration-dependent attenuation of FFN246 uptake strongly supports the conclusion that SERT is the primary gateway through which FFN246 enters HEK293-SERT cells, and that its transport can be pharmacologically manipulated in a predictable and reproducible manner. Collectively, these findings validate FFN246 as a specific SERT substrate, lending confidence to its potential utility as a fluorescent tool for investigating serotonergic transport dynamics in future pharmacological studies.

Taken together, the uptake inhibition data presented in Figures 4 and 5 establish a strong foundation for the utility of FFN246 as a reliable fluorescent tool for studying SERT-mediated serotonin influx, while also demonstrating the capacity of fluoxetine to modulate this process in a concentration-dependent manner. However, a critical question remained unanswered at this stage; could FFN246 be equally effective at capturing efflux dynamics, that is, the movement of a substrate out of the cell through SERT, and if so, could it serve as a practical readout for identifying drug candidate capable of inducing efflux? This is an important distinction, as SERT-mediated efflux represents a fundamentally different pharmacological phenomenon from reuptake inhibition, and one that remains comparatively underexplored as a therapeutic target. The ability to quantify efflux using a fluorescence-based approach would open the door to higher-throughput screening of candidate compounds, potentially accelerating the identification of novel therapeutics for serotonin-related disorders, including strategies focused on drug repurposing, where existing compounds are evaluated for previously unrecognized mechanisms of actions at serotonergic targets (Griebel, 1995).

To begin exploring the efflux capabilities of FFN246, amphetamine was selected as the primary pharmacological tool, given its well-characterized ability to induce reverse transport of both dopamine and serotonin through their respective monoamine transporters (Khaliq, 2005; Rudnick, 1992). In contrast to reuptake inhibitors like fluoxetine, which simply block the transporter, amphetamine actively triggers the transport to run in reverse, pushing the monoamines out of the cell rather than allowing them to be taken back up. This made it an ideal first candidate for testing whether FFN246 could serve as a fluorescent readout of efflux. However, the initial efflux assay yielded a surprising result, despite testing amphetamine across concentration range of 1 uM, 3 uM, 10 μM, 30 μM, 100 μM, and 300 μM, no detectable FFN246 efflux was observed in the supernatant fraction. Furthermore, direct measurement of cellular fluorescence following supernatant collection revealed no meaningful reduction in intracellular FFN246 signal relative to the negative control, providing no indication that

FFN246 had been transported out of the cells under any of the conditions tested (Figure 6).

Repeated iterations of the efflux assay incorporating varied time frames, FFN246 concentrations, and measurement approaches, including parallel assessment of both the supernatant and cellular fractions, consistently failed to produce detectable efflux signal across all conditions tested (Figure 7 & 8). This persistent null result prompted a review of the broader FFN literature, which offered a plausible explanation: fluorescent neurotransmitter analogs such as FFN246 may become sequestered within cells through interactions with hydrophobic intracellular regions and proteins, effectively trapping the compound and preventing its release through SERT-mediated reverse transport (Choi, 2019).

Sodium ion concentrations represent an additional complicating factor, as intracellular sodium levels are known to influence SERT conformation in ways that may impair its capacity to mediate reverse transport (Khoshbouei, 2003). It is therefore possible that FFN246, while readily taken up via SERT, becomes sequestered within intracellular compartments rather than being efficiently released back into extracellular space. Compounding this uncertainty, any FFN246 that does exit the cell through alternative non-SERT pathways may be indistinguishable from background signal relative to the loading control. Ultimately, whether the absence of detectable efflux reflects true intracellular sequestration, leakage through non-specific pathways, or a combination remains an open question, one that will require more targeted experimental approaches to resolve.

Following the inconclusive efflux results with amphetamine, attention shifted toward examining the cellular conditions that might be necessary to facilitate SERT-mediated reverse transport of FFN246. Prior work by Khoshbouei et al. demonstrated that elevated intracellular sodium concentrations promote dopamine efflux, suggesting that ionic environment within the cell plays a meaningful role in determining the directionality of monoamine transport. Building on this rational, ouabain was selected as a pharmacological tool to manipulate intracellular sodium levels, by inhibiting the NA+/K+ ATPase, ouabain blocks the pump responsible for maintaining sodium gradients across the cell membrane, leading to an accumulation of intracellular sodium that may be permissive for reverse transport through SERT (Sandtner, 2011).

To test this hypothesis, ouabain was applied at a fixed concentration of 100 μM across the full FFN246 concentration range (0.5 μM, 2.5 μM, 5 μM, 10 μM, and 25 μM) to assess its impact on both uptake and efflux dynamics. Contrary to expectations, ouabain treatment produced no discernible effect on FFN246 uptake relative to untreated controls, suggesting that NA+/K+ ATPase inhibition alone is insufficient to meaningfully alter SERT-mediated influx under these experimental conditions (Figure 9). Similarly, co-

administration of ouabain with amphetamine across concentrations of 1 μM, 10 μM, and 100 μM failed to produce detectable FFN246 efflux, indicating that even the combined strategy of elevating intracellular sodium while simultaneously engaging amphetamine-induced reverse transport was not sufficient to overcome the apparent sequestration of FFN246 within the cell (Figure 10).

A further limitation of the current experimental model is that supernatant fluorescence reflects the net movement of FFN246 across the entire cell population rather than efflux from individual cells (Sitte, 2000). Consequently, any FFN246 released by one cell in response to amphetamine treatment could potentially be taken up by a neighboring cell within the 10-minute assay window, effectively masking true efflux and contributing to the consistently low supernatant signal observed across experiments.

One promising approach to circumventing this limitation would be the adoption of superfusion experimental model, in which a continuous flow of buffer over the cell monolayer would carry released FFN246 away from the cells immediately upon efflux, effectively eliminating the opportunity for neighboring cell reuptake and providing more accurate representation of true SERT-mediate release (Sitte, 2000; 2001). Superfusion assays have an established track record in monoamine transport research and would represent a logical and well-supported next step in evaluating the full utility of FFN246 as an efflux detection tool.

Collectively, the present findings demonstrate that FFN246 is a reliable and specific tool for tracking SERT-mediated serotonin uptake dynamics, as evidenced by its concentration-dependent accumulation in HEK293-SERT cells and its sensitivity to fluoxetine-induced SERT inhibition. However, its utility as a quantitative readout of serotonin efflux remains unresolved under the current experimental conditions. Whether this reflects true intracellular sequestration following SERT-mediated uptake, passive leakage through alternative membrane channels or non-selective transporters, or a methodological limitation of the bulk fluorescence measurement approach remains to be determined. Addressing these questions through more targeted experimental designs, including superfusion assays, single-cell imaging approaches, or direct pharmacological manipulation of intracellular compartmentalization will be essential for fully establishing the scope of FFN246's utility as a dual influx-efflux reporter and advancing its potential as a screening tool for novel serotonergic therapeutics.

Conclusion

The present study evaluated the utility of the false fluorescent neurotransmitter FFN246 as a tool for investigating serotonin transport dynamics in a SERT-expressing cellular model. FFN246 demonstrated clear promise as a specific and reliable reporter of SERT-mediated uptake, evidenced by its concentration-dependent accumulation in HEK293-SERT cells and its sensitivity to pharmacological SERT inhibition of fluoxetine. These findings establish a validated fluorescence-based framework for studying serotonin influx that may prove valuable in future high-throughput screening applications

However, the capacity of FFN246 to model serotonin efflux dynamics remains unresolved. Despite systematic efforts to induce reverse transport through amphetamine administration and manipulation of the intracellular sodium gradient via ouabain, no detectable efflux was observed under any conditions tested. Intracellular sequestration, leakage through alternative transport pathways, and conformational constraints on SERT-mediated reverse transport represent the most plausible explanations for this outcome, though distinguishing between these mechanisms will require more targeted experimental approaches such as superfusion assays or single-cell imaging strategies.

Altogether, this work advances our understanding of the capabilities and current limitations of FFN246 as a serotonergic imaging tool and lays a meaningful foundation for future investigations aimed at fully characterizing its transport dynamics. Continued research of FFN246-based assays holds genuine promise for broadening our mechanistic understanding of serotonin neurotransmission and its relevance to the development of novel therapeutics for neuropsychiatric disorders.

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