MASTERARBEIT | MASTER'S THESIS

Titel | Title

Role of Synucleins in Modulation of Monoamine Transporters

verfasst von | submitted by

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angestrebter akademischer Grad | in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien | Vienna, 2024

Studienkennzahl lt. Studienblatt | Degree programme code as it appears on the student record sheet:	UA 066 830
Studienrichtung lt. Studienblatt | Degree programme as it appears on the student record sheet:	Masterstudium Molecular Microbiology, Microbial Ecology and Immunobiology
Betreut von | Supervisor:	Mag. Thomas Steinkellner PhD

Acknowledgements

My gratitude goes to Thomas Steinkellner for offering me a position in his lab. This has been one of the most exciting accomplishments in my life. Your support and personal guidance will always be appreciated. I consider myself lucky to have played a part in your scientific endeavors and wish you all the best as you journey forward. Again, my sincerest thanks.

A special thanks to Marko Roblek for teaching me the ropes. You were a great mentor throughout this project and a lot of credit goes to you – We keep in touch man!
I also want to acknowledge Sonja Sucic. You are such a kind, helpful, and thoughtful person - you deserve many thanks. Ali, this acknowledgement includes you too – You were always there to help, and I appreciate you. Fatimah, you are a lovely lab assistant, and your work deserves recognition. Thank you.

I want to thank my lab mates Siva, Sofia, Segolene, Amila, Alex, Iina, Fabian, (Nikita and Shima too!). Ya'll are fun, supportive, and just awesome. Thanks for the laughs and all the memories we shared together – both inside and outside of the lab. I enjoyed every minute of it.

Alejandro, Paulo, Sunny, Klaus, Alex, David, and Fatinah, each of you are special and you have made living life in Vienna even more special. I deeply value of our friendship.

My biggest gratitude goes to my parents, I love you both so much and miss you daily. To my cousins David, Gabe & Isaac; thanks for the encouragement and emotional support along the way - You guys are dearly loved and I'm excited to celebrate this academic achievement with you soon. You too Sami!

Anna, this thesis is for us. I love you.

Abstract

Synucleins (α-Syn, β-Syn, and γ-Syn) are small proteins involved in the regulation of neurotransmission within the nervous system. Previous work in the Steinkellner lab demonstrated that α-Syn overexpression increases cell surface expression of serotonin transporters (SERT), thereby amplifying serotonin reuptake in a α-Syn-dependent manner. However, the roles of β-Syn & γ-Syn in the regulation of monoamine transporters (MATs) remain largely unexplored, providing a focus for this thesis.

This study aims to determine whether β-Syn and γ-Syn modulate MATs in a similar manner to α-Syn or exhibit distinct regulatory roles. Through overexpression experiments in cultured cell lines, I found that although β-Syn and γ-Syn colocalized with MATs, neither affected the cell surface expression of these transporters nor the reuptake of their respective monoamines. To assess α-Syn's influence on SERT-mediated reuptake, I conducted gene silencing experiments. Using small interfering RNAs (siRNAs) to reduce endogenous α-Syn expression and generating a CRISPR-Cas9-mediated *SNCA* knockout in a cell line, I confirmed that reduction or loss of α-Syn negatively impacted SERT activity and expression. This contrasts with the effects of α-Syn overexpression, highlighting the regulatory role α-Syn has on SERT function.

These findings provide insights into differential regulation of monoaminergic neurotransmission by synucleins, suggesting that α-Syn uniquely modulates MAT activity by affecting cell surface expression and reuptake, while β-Syn and γ-Syn do not exhibit similar effects.

Zusammenfassung

Synucleine (α-Syn, β-Syn und γ-Syn) sind kleine Proteine mit wichtigen Funktionen bei der Regulation der Neurotransmission. Vorangegangene Experimente des Steinkellner Labors demonstrierten, dass eine α-Syn Überexpression die Expression von Serotonintransportern (SERT) auf der Zelloberfläche erhöht und dadurch die Serotoninwiederaufnahme in einem α-Syn-abhängigen Mechanismus steigert. Die Rolle von β-Syn und γ-Syn bei der Regulierung von Monoamintransportern (MATs) bleibt jedoch noch weitgehend unerforscht, weshalb dies den Schwerpunkt dieser Arbeit bildet.

Diese Studie zielt darauf ab, festzustellen, ob β-Syn und γ-Syn die MATs auf ähnliche Weise wie α-Syn modulieren oder unterschiedliche regulatorische Rollen ausüben. Ich konnte durch Überexpressionsexperimente in kultivierten Zelllinien herausfinden, dass β-Syn und γ-Syn zwar mit MATs kolokalisierten, aber weder die Zelloberflächenexpression dieser Transporter noch die Wiederaufnahme ihrer jeweiligen Monoamine beeinflussten. Um den Einfluss von α-Syn auf die SERT-vermittelte Wiederaufnahme zu untersuchen, habe ich Gen-Silencing Experimente durchgeführt. Unter Verwendung kleiner, interferierender RNAs (siRNAs) zur Reduzierung der endogenen α-Syn-Expression und zur Erzeugung eines CRISPR/Cas9-vermittelten *SNCA* (= Gen von α-Syn) -Knockouts in einer humanen Zelllinie, konnte ich bestätigen, dass die Verringerung oder der Verlust von α-Syn die SERT-Aktivität und Expression negativ beeinflusste. Darüber hinaus reduzierte der *SNCA*-Knockout die maximale Geschwindigkeit der Serotoninaufnahme, ohne die Affinität von SERT für Serotonin wesentlich zu verändern. Dies steht im Gegensatz zu den Auswirkungen der Überexpression von α-Syn und unterstreicht die regulatorische Rolle, die α-Syn auf die SERT-Funktion ausübt.

Diese Ergebnisse liefern Einblicke in die differenzierte Regulation der monoaminergen Neurotransmission durch Synucleine und legen nahe, dass α-Syn die MAT-Aktivität auf einzigartige Weise moduliert, indem es die Zelloberflächenexpression und Wiederaufnahme beeinflusst, während β-Syn und γ-Syn diese Effekte nicht zeigen.

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1. Introduction

1.1 Overview

In his reflective autobiographic paper titled *Journey of a late blooming biochemical scientist*, Julius Axelrod described the study of chemical neurotransmission as having a "colorful and unusual history". While his insights extend beyond the immediate scope of this thesis, they provide valuable historical context for understanding the foundational experiments that established the mechanisms of neurotransmission and neurotransmitter inactivation, particularly through uptake processes at nerve terminals (Axelrod, 2003; Hertting & Axelrod 1961). Axelrod's research laid the groundwork for current studies exploring interactions between synucleins and MATs in modulation of neurotransmission with potential implications for neurodegenerative diseases. This introduction also contextualizes contemporary findings in line with the outcomes of my thesis work.

The synuclein family comprises three small, highly conserved proteins characterized by their sequence homology **(Fig. 1)** and structural similarities (Jakes et al., 1994; Surguchov, 2008). These proteins, predominantly localize in presynaptic terminals of neurons and are implicated in regulating monoaminergic neurotransmission and maintaining proper synaptic function (Surguchov, 2008; Lavedan, 1998; Galvin et al., 2001).

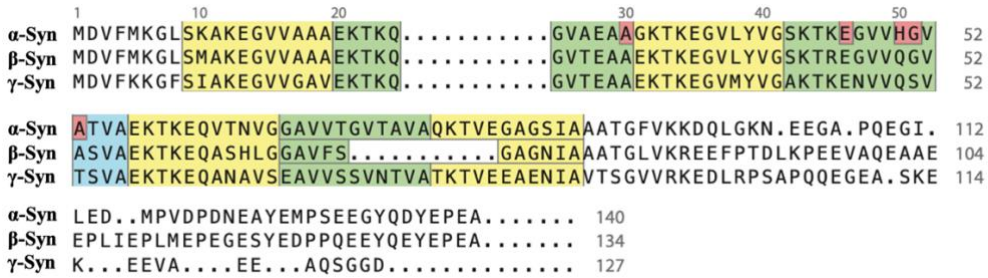


Figure 1: Sequence alignment of human α, β and γ-Syn. Figure adapted from Runwal and Edwards, 2021.

Synucleins contribute to various physiological processes, including synaptic plasticity, the regulation of neurotransmitter release and reuptake (Clayton and George, 1998; George, 2002), and their imbalance may affect neuronal survival (Carnazza et al., 2022). However, under pathological conditions, particularly in Parkinson's disease (PD), α-Syn can misfold and aggregate in the presynaptic region (Spillantini et al., 1997). This may contribute to disruptions in monoamine signaling and give rise to neurodegenerative diseases like Alzheimer's disease and dementia (Kruger et al., 1998; Bourdenx et al., 2017).

Understanding how synucleins interact with MATs (with a focus on SERT and DAT throughout this thesis) is essential for distinguishing their normal physiological roles from their pathological effects. MATs regulate neurotransmitter levels at the synapse by facilitating the reuptake of their respective monoamines (Nelson, 1998), a vital process for terminating synaptic-signaling and maintaining neurotransmitter homeostasis (Blakely and Edwards, 2012). Consequently, MATs serve as key pharmacological drug targets for modulating neurotransmitter uptake and release (Torres et al., 2003). A detailed review by Sidhu et al. (2004) highlights significant potential implications of disrupted DAT regulation by α-Syn in PD and other synucleinopathies.

α-Syn has been shown to physically interact with DAT and modulate the activity and trafficking of these transporters, directly impacting synaptic function (Lee et al., 2001; Wersinger & Sidhu, 2003; Oaks & Sidhu, 2013). Previous work suggested that α-Syn can also modulate SERT activity, leading to enhanced serotonin (5-HT) reuptake without altering the transporters affinity for 5-HT (Sofic, 2024). However, the effects of α-Syn overexpression on MATs appear to vary depending on experimental conditions, either reducing or significantly increasing the rate of dopamine (DA) uptake by altering cell surface availability (Wersinger and Sidhu, 2003; Swant et al., 2011; Butler et al., 2015; Lee et al., 2001).

While the regulatory influence of α-Syn on MAT activity has been well-documented, the roles of β-Syn and γ-Syn are less understood. Butler et al. (2017) describes α-Syn's involvement in regulation of DA neurotransmission in detail. Several studies indicate that each synuclein may have distinct regulatory influences on the different MATs (reviewed by Oaks and Sidhu, 2011). For instance, γ-Syn can modulate the availability of serotonin through interaction with SERT, while β-Syn does not appear to regulate SERT activity (Wersinger and Sidhu, 2009). Furthermore, β-Syn and γ-Syn modulate DAT trafficking through mechanisms independent of the microtubule-based transport, distinguishing their regulatory pathways from that of α-Syn and impacting distribution of DAT to the cell surface (Oaks et al., 2013). Additionally, β-Syn may provide a protective or compensatory effect against α-Syn pathology by reducing its membrane binding and subsequent aggregation (Burré et al., 2015; Carnazza et al., 2022).

1.2 The Monoaminergic System

1.2.1 Monoaminergic Pathways

The monoaminergic system is a collective of neural pathways that provide essential structural organization within the brain. These pathways originate in specific nuclei and project to various regions, forming the framework for neurotransmission. They are responsible for regulating critical physiological functions and behaviors using the modulatory neurotransmitters dopamine, norepinephrine, and serotonin. Dopaminergic neurons arise from the substantia nigra pars compacta (SNc) and ventral tegmental areas (VTA) of the midbrain, projecting to the striatum (caudate nucleus and putamen), the limbic system (including the amygdala, hippocampus, and nucleus accumbens), and prefrontal cortex (Kandel et al., 1991; Ogawa et al., 2014 and 2018). These projections are essential for motor control, motivation, and reward-driven behavior. Serotonergic neurons, on the other hand, originate in the raphe nuclei of the brainstem and innervate widespread brain regions. These include the amygdala, thalamus, hippocampus, prefrontal cortex, and limbic systems, where they influence mood, emotion, sleep, and appetite (Hornung, 2003). The serotoninergic system is closely linked to mood regulation and emotional responses, and hence provides a major target for antidepressants (Torres et al., 2003; Taylor et al., 2004)

1.2.2 Monoamine Neurotransmission

Monoamines are biosynthesized from aromatic amino acid precursors in a two-step process. For example, phenylalanine is hydroxylated to tyrosine, which is then converted into 3,4-dihydroxy-phenylalanine (L-DOPA) by tyrosine hydroxylase and further into DA through action of L-aromatic amino acid decarboxylase (Best et al., 2009). Similarly, 5-HT is synthesized from tryptophan, which is hydroxylated to form 5-hydroxytryptophan before being decarboxylated to 5-HT (Siegel et al., 1999; Best et al., 2010).

Under physiological conditions, vesicular monoamine transporters (VMATs) concentrate monoamines into synaptic vesicles, which prevents MA oxidation in the cytosol due to a more acidic environment (Parsons, 2000; Guillot and Miller., 2009). These vesicles also protect MAs from degradation in the cytosol and are required for synaptic release (Liu et al., 1992; Erickson et al., 1992; Guillot and Miller., 2009). This unloading of vesicular content into the synaptic cleft occurs through synaptic vesicle exocytosis (Südhof and Rizo 2011), a process initiated by an action potential that depolarizes the cell membrane and opens voltage gated calcium

channels. The increase in intracellular calcium concentration then drives the binding of SVs to the presynaptic membrane via soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, including synaptobrevin (also known as vesicle-associated membrane proteins (VAMPs), syntaxin-1A and SNAP-25 (Söllner et al., 1993; Pevsner et al., 1994; Augustine et al., 1987; Südhof, 2004). These proteins form the SNARE-complex, enabling vesicle fusion to membranes and allow neurotransmitter release (Yan et al., 2022). Once released, MAs bind their respective receptors on the pre- and post-synaptic membranes to initiate downstream signaling pathways.

Secondary active transporters such as the MATs are then responsible for retrieving neurotransmitters from the synaptic cleft back into the presynaptic neuron, allowing for recycling or degradation. This reuptake inactivates neurotransmission and potentially prevents neurotoxicity occurring from excess neurotransmitter accumulation (Giros et al., 1996; Nelson 1998; Amara et al., 1998; Iversen 1971; Südhof, 2004). Additionally, enzymes like monoamine oxidases maintain physiologically appropriate levels by degrading excess monoamines (Männistö and Kaakkola, 1999; Youdim et al., 2006).

1.2.3 Monoamine Transporters

MATs, including DAT, SERT and norepinephrine transporter (NET), are part of the sodium dependent solute carrier 6 (SLC6) family of proteins. They are encoded by the human *DAT (SLC6A3), SERT (SLC6A4) and NET (SLC6A2)* genes, which map to chromosome 5p15.3 and 17q11.2 and 16q12.2 respectively (Ramamoorthy et al., 2011). DAT, SERT and NET share structural similarities, characterized by 12 transmembrane alpha-helical domains (620-630 residues) and cytosolic-facing N and carboxy (C) termini (Blakely and Edwards, 2012; Kristensen et al., 2011). DAT has three extracellular N-linked glycosylation sites, and depending on glycosylation, exhibits a molecular weight of around 60-100 kD that can form oligomeric complexes (Bjerggaard et al., 2004; Sitte and Freissmuth, 2003; Li et al., 2004; Amara and Kuhar, 1993).

These transporters are widely expressed in brain regions corresponding to their associated monoaminergic neurons and are predominately localized to the cell surface of presynaptic terminals (Hoffman et al., 1998; Sur et al., 1996; Ciliax et al., 1999). MATs regulate the bidirectional transport of neurotransmitters across biological membranes, primarily through a sodium-dependent reuptake mechanism. This transport is driven by the sequential binding and

co-transport of substrate molecules with Na^+/Cl^- ions (Nelson 1998; Kristensen et al., 2011; Amara and Kuhar, 1993), established through activity of the Na^+/K^+ ATPase.

Disruption in monoamine neurotransmission, due to altered MAT function, may contribute to neuropathological and psychiatric disorders, such as PD and ADHD (Oaks and Sidhu, 2011; da Silva et al., 2023). Selective inhibitors of SERT, such as paroxetine, inhibit reuptake and alter levels of neurotransmitters in the synapse. They are widely used as antidepressants (Torres et al., 2003). MATs are also capable of releasing monoamines, a reverse transport process induced by psychostimulants like amphetamines (Khalig et al., 2005; Swant et al., 2011; Kristensen et al., 2011). The presence of MATs at the cell membrane is crucial for efficient monoaminergic signaling, the reuptake of extracellular monoamines, and replenishments of synaptic vesicles (Worrall & Williams, 1994; Zahniser & Doolen, 2001). Their activity and cell surface expression is tightly regulated by endocytic trafficking, and can be influenced by direct interactions with α-Syn (Bu et al., 2021; Oaks and Sidhu, 2011).

1.2.4 α-Syn Regulation of Monoamine Homeostasis

Synucleins belong to a family of small proteins that influence monoaminergic neurotransmission by regulating key processes including biosynthesis, storage, release, and reuptake (Venda et al., 2010; Oaks and Sidhu, 2011). Among them, α-Syn plays a role in maintaining neurotransmission. α-Syn has been identified as a direct binding partner of DAT, modulating its activity, and influencing DA reuptake (Lee et al., 2001). It also interacts with tyrosine hydroxylase, the enzyme responsible for DA synthesis, thereby regulating the initial stages of dopamine production (Perez et al., 2002; Murphy et al., 2000). Beyond synthesis and reuptake, α-Syn is involved in SV dynamics by facilitating both endocytosis and exocytosis (Bendor et al., 2013; Südhof et al., 2011). It promotes the fusion of SVs with the plasma membranes through its role in assembling the SNARE-complex at the synapse (Südhof, 2014; Bonini and Giasson, 2005). Specifically, α-Syn interacts directly with VAMP2 via its C-terminus, while the N-terminus tethers itself to the plasma membrane (Burré et al., 2010; Logan et al., 2017; Wang et al., 2016). These interactions are critical for efficient neurotransmitter release and recycling.

1.3 The Synuclein Family

Synucleins are intrinsically disordered proteins, meaning they lack a stable three-dimensional structure under physiological conditions. Depending on post-translational modifications, synucleins are small proteins approximately 14-19 kD in size (Jakes et al., 1994; Weinreb et al., 1996) and are abundantly expressed throughout the central nervous system. They appear in three different isoforms; α, β and γ, encoded by the *SNCA, SNCB and SNCG* genes, which map to chromosome 4q21, 5q35, and 10q23 respectively (George, 2002). Historically, synuclein was first isolated from the Pacific Electric Ray - *Torpedo Californica* (Maroteaux et al., 1988), and based on its amino acid sequence is generally considered to be γ-Syn. α-Syn was later identified in amyloid plaques of brains from patients with Alzheimer's disease (Uéda et al., 1993) and soon after, β-Syn was extracted from human brain tissue (Jakes et al., 1994).

All three synucleins share an affinity for highly curved lipid membranes, and often colocalize near synaptic vesicles (Maroteaux etl al., 1988; Davidson et al., 1998; Lavedan, 1998; Burré et al., 2015). This affinity plays a crucial role in their function, particularly in the regulation of synaptic transmission and their localization at presynaptic sites (Jensen et al., 2011; Middleton and Rhoades, 2010). α-Syn and β-Syn proteins are predominantly expressed in the central nervous system and localized in presynaptic regions, while γ-Syn is widely distributed in the cytosol and less specific to the brain (Uéda et al., 1994; Lavedan, 1998; Surguchov et al., 2001). Despite their structural similarities each synuclein isoform exhibits distinct biological functions and pathological implications, particularly in neuronal tissues.

1.3.1 Structural Organization and Behavior

The synucleins share considerable amino acid sequence homology, especially between α-Syn and β-Syn. Respectively, α-Syn consists of 140, β-Syn of 134 and γ-Syn of 127 amino acids. All synucleins share a common structural motif, characterized by three functionally distinct regions: an amphipathic lysine-rich N-terminus, a central hydrophobic non-amyloid-β component (NAC), and a negatively charged C-terminus (Fusco et al., 2014) (also *cf.* **Fig. 1**). Each isoform contains multiple imperfect repeats of the alpha-helical lipid-binding motif "KTKEGV", which is highly conserved in the N-terminal domain (Jakes et al., 1994). The N-terminus modulates synuclein binding to phospholipid membranes, the NAC domain promotes aggregation into β-amyloid fibrils, and the C-terminus is largely unfolded and mediates binding to membranes and chaperones (Ueda et al., 1993; Giasson et al., 2001; Yoshimoto et al., 1995;

Surguchov, 2015). These regions collectively contribute to the ability of synucleins to either promote or inhibit aggregation.

α-Syn is estimated to constitute 1.0% of the total cytosolic neuronal proteins (Iwai et al., 1995; Stefanis et al., 2012) and is normally concentrated at presynaptic terminals of neurons in the brain (Iwai et al., 1995; Jakes et al., 1994; Totterdell et al., 2004). The C-terminus is particularly responsible for α-Syn's cytotoxicity and aggregation (Zhang et al., 2022). In presynaptic terminals, α-Syn fluctuates between a soluble state and being bound to membranes including synaptic vesicles (Burré, 2015; Carnazza et al., 2022). In the cytosol, it predominately exists as an unstructured monomer that adopts an alpha-helical structure when bound to lipid membranes (Fauvet et al., 2012; Kahle et al., 2000; Jain et al., 2018; Uversky et al., 2002). α-Syn can deviate from its normal soluble or lipid-bound state into oligomeric species that can aggregate and cause neurotoxic effects (Uversky, 2007; Takeda et al., 1998; Sode et al., 2006). However, endogenous α-Syn has been shown to fold into tetrameric form (58 kD) in the cytosol and may withstand the effects of aggregation (Bartels et al., 2011). Similarly, β-Syn has been shown to form tetramers (60 kD), although these structures destabilize into monomers due to lysis sensitivity (Dettmer et al., 2013).

β-Syn has a high content of negatively charged residues that contribute to its less compact conformation. Unlike α-Syn and γ-Syn, β-Syn lacks a majority of its NAC domain (Jakes et al., 1994; Uversky et al., 2002; Burré et al., 2018), which may explain its reduced propensity to aggregate and form amyloid fibrils (Williams et al., 2018; Goedert, 2001). γ-Syn is the least studied member of the synuclein family, although it shares structural similarities with α-Syn. However, it lacks three conserved tyrosine residues in the C-terminal, a feature found in both α-Syn and β-Syn, which may contribute to γ-Syn's distinct functional roles and susceptibility to oxidative stress (Uversky et al., 2002; Surguchov, 2008). Unlike α-Syn, γ-Syn is more closely associated with certain cancers rather than neurodegenerative diseases, as it does not aggregate as readily (Ji et al., 1997; Surguchov, 2008).

1.3.2 Functional Variation

All three synucleins are implicated in synaptic transmission and homeostasis, primarily through their roles in endocytosis, exocytosis, vesicle cycling (Burré, 2015; Liu et al., 2021; Runwal and Edwards, 2021). Although their precise physiological roles remain elusive, synucleins are thought to be crucial for maintaining synaptic homeostasis and neurotransmission. Variations

in protein expression levels and distribution throughout the brain further differentiate their functional roles (Carnazza et al., 2022).

Studies using triple knockout mice have shown modest effects on dopaminergic system dysregulation (Greten-Harrison et al., 2010), suggesting that synucleins may compensate for one another's functional deficits. This compensatory mechanism might help maintain normal synaptic function when α-Syn is either overexpressed or downregulated (Alarcón-Arís et al., 2018). The ability of the synucleins to mutually compensate for functional losses could be key to understanding how they interact to regulate synaptic transmission and prevent pathological aggregation (Hashimoto et al., 2001; Masliah et al., 2000; Uversky et al., 2003).

In addition to its synaptic presence, α-Syn's has been detected in the nucleus, where it may bind to double-stranded DNA and potentially contribute to cell death by regulating DNA repair mechanisms (Goers et al., 2003; Yu et al., 2007; Shaser et al., 2019). Although this nuclear role is not fully understood, it opens new avenues for exploring α-Syn non-synaptic functions. α-Syn is also expressed in non-neuronal cells, including red blood cells which further suggests diverse roles beyond the brain (Nakai et al., 2007; Barbour et al., 2008).

In contrast to α-Syn, little is known about the physiological or pathological function of β-Syn and γ-Syn, particularly their roles in modulating SERT cell surface expression, localization, and trafficking. γ-Syn is not exclusively cytosolic and tends to favor the peripheral nervous system more than specific brain regions. It is also expressed by glia cells and dopaminergic neurons, with some studies linking γ-Syn to certain cancers (Galvin et al., 2001; Inaba et al., 2005). This broader distribution pattern suggests γ-Syn may have roles outside the central nervous system (Lavedan et al., 1998; Surgucheva et al., 2006). Like α-Syn, γ-Syn can bind to synaptic vesicles; however, it's C-terminal domain is unable to bind synaptobrevin/VAMP2 and does not affect SNARE-complex assembly (Ninkina et al., 2012). Some evidence suggests that γ-Syn an β-Syn and may modulate α-Syn ability to bind SVs, thus influencing α-Syn's physiological activities at the neuronal synapse (Carnazza et al., 2022).

β-Syn, acts as molecular chaperone to mitigate the toxic effects of α-Syn aggregation by inhibiting fibrillation (Uversky et al., 2002; Jain et al., 2018). This chaperone activity helps maintain dopamine regulation and protects neurons from damage (Barba et al., 2022). In addition to its chaperoning role, β-Syn regulates synaptic function, lipid binding,

neurotransmission, and participates in protein degradation pathways and apoptosis (Hayashi and Carver, 2022). Interestingly, β-Syn may enhance VMAT-2-dependent dopamine uptake by striatal synaptic vesicles, a function unique to β-Syn (Ninkina et al., 2021). Collectively, these roles contribute to maintain cellular homeostasis and protect against neurogenerative conditions.

1.3.3 Synuclein Modulation of MATs

All three synucleins are known to modulate the trafficking, activity, and surface expression of MATs *in vitro* (Oaks and Sidhu, 2011; Sidhu et al., 2004; Butler et al., 2017). A key feature of this modulation is the protein-protein interaction between the NAC region of α-Syn and the C-terminus of DAT, which affects the cell surface distribution and availability of the transporter (Lee et al., 2001). This interaction influences the magnitude of MAT-mediated neurotransmitter reuptake (Oaks & Sidhu, 2011). Most α-Syn-MAT complexes are localized at the plasma membrane of monoaminergic neurons and may have significant implications for neurodegenerative diseases (Butler et al., 2015; Baba et al., 1998)

Overexpression of α-Syn has been shown to increase SERT reuptake activity, in co-transfected N2a cells (Sofic, 2024). Some studies report a reduction in reuptake activity due to decreased trafficking of DATs to the cell surface (Wersinger et al., 2006; Wersinger & Sidhu, 2003) while another indicated an increase in reuptake activity (Lee et al., 2001). Additionally, an 80% knockdown of α-Syn reduced DAT activity by 50% in human neuronal cell lines, accompanied by a decrease in the cell surface expression of hDAT (Fountaine and Wade-Martins, 2007; Fountaine et al., 2008). These findings suggest that α-Syn plays a critical role in regulating MAT trafficking and reuptake activity, and disruptions in this process may contribute to neurodegenerative pathologies.

While the modulatory effects of α-Syn on MATs are well documented, the roles of β-Syn and γ-Syn in MAT regulation are an ongoing topic of research. Despite lacking the NAC domain, overexpression of β-Syn has been shown to influence MAT activity (Oaks and Sidhu, 2011; Sidhu et al., 2004; Oaks et al., 2013). Recent research suggests that γ-Syn transcription levels in SNc/VTA regions may regulate DAT in a similar manner to α-Syn (Pavia-Collado et al., 2022). Disruption of this regulatory process may contribute to a toxic increase in DA reuptake, leading to the accumulation of α-Syn aggregates, which are implicated in neurogenerative diseases (Sidhu et al., 2004).

1.3.4 Synucleinopathies

Structural differences between the synucleins contribute to their distinct functional roles under normal conditions. However, the abnormal accumulation of monomeric α-Syn can lead to formation of insoluble amyloid fibrils, which aggregate into larger inclusions such as Lewy bodies (Farrer 2008; Masliah et al., 2001; Burré et al., 2018; Goedert et al., 1997). The process of amyloid fibril formation involves nucleation, growth, elongation, and secondary nucleation (Buell et al., 2014). Once α-Syn aggregates into stable β-sheets rich amyloid fibrils, it becomes highly resistant to disaggregation. Smaller soluble oligomeric α-Syn species, also known as prefibrillar aggregates, are believed to induce cytotoxicity and may potentiate disease (Emin et al., 2022).

Lewy body progression is highly characteristic of synucleinopathies, and their presence supports a clinical diagnosis of Parkinson's disease (Spillantini et al., 1997; Goedert, 2013). PD is characterized by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta, which leads to motor deficits such as resting tremor and bradykinesia in aging populations (Bernheimer et al., 1973; Camerucci et al., 2021). The onset of PD typically occurs in individuals around 70 years of age, although early signs may appear as early as 50 (Camerucci et al., 2021; Pang et al., 2019). As PD progresses, misfolded α-Syn has been hypothesized to move from one cell to the next in a staging process hypothesized by Braak et al. (2003a). This propagation of Lewy body pathology moves from the brainstem to higher cortical regions in a prion-like manner (Uchichara and Glasson, 2016; Goedert et al., 2017), contributing to the widespread degeneration of dopaminergic neurons within the SNc as well as other monoamine neurons. The loss of monoaminergic function in specific brain regions contributes to the onset of depression in approximately 40% of PD patients, often preceding motor symptoms (Allain et al., 2000; Maletic et al., 2007).

Several rare mutations and gene multiplications in the *SNCA* gene are linked to familial forms of PD. The first significant α-Syn mutation, A53T, was identified in 1997 (Polymeropoulos et al., 1997). This event marked a breakthrough in understanding of PD's genetic origins. Since then, additional *SNCA* mutations such as E46K and A30P have been discovered, and these mutations are known to increase aggregative behavior of α-Syn (Polymeropoulos et al., 1997; Zarranz et al., 2004; Kruger et al., 1998). Improper folding of α-Syn leads to β-sheet-rich amyloid aggregates, which may originate in the gastrointestinal tract and spread to the CNS via the vagus nerve (Braak et al., 2003b; Goedert et al., 2017; Borghammer et al., 2018).

Horsagar et al. (2020) reported that α-Syn pathology can spread via two different routes: either originating in the brain and spreading to the gut, or vice versa, based on the initial motor symptoms of PD patients. This gut-brain connection and the toxicity associated with α-Syn and migration are supported by both *in vitro and in vivo* with clinical evidence (Rietdijk et al., 2017).

Although β-Syn and γ-Syn do not accumulate in Lewy pathology (Spillantini and Goedert, 2018), their roles in neurodegeneration are not fully understood. Interestingly, β-Syn has been shown to induce significant degeneration of DA neurons, particularly in the presence of DA or oxidized metabolites, suggesting a role in PD (Raina A., 2021; Taschenberger et al., 2013). Mutations in β-Syn, such as P123H and V70M, have been linked to neurodegenerative diseases like dementia with Lewy bodies, where lysosomal pathology plays a role (Ohtake et al., 2004; Wei et al., 2007). Additionally, it has been associated with multiple sclerosis and contributes to gray matter damage (Lodygin et al., 2019).

1.3.5 Summary

Given the potential for β-Syn and γ-Syn to exhibit distinct or complimentary functions relative to α-Syn, this thesis attempts to understand their specific roles in modulating MAT activity *in vitro*. By investigating synucleins effects on the cell surface expression and function of MATs in both neuronal and non-neuronal cells, this research seeks to uncover unique and shared mechanisms through which each synuclein regulates monoamine reuptake. Biochemical methods were employed under conditions of both overexpression and gene silencing to explore the regulatory roles of synucleins in the monoaminergic system. Ultimately, this thesis seeks to deepen our understanding of synuclein biology. These insights could inform future therapeutic strategies that target synuclein-mediated disruptions in synaptic signaling, revealing how their dysregulation contributes to neurodegenerative diseases like PD.

1.4 Aims

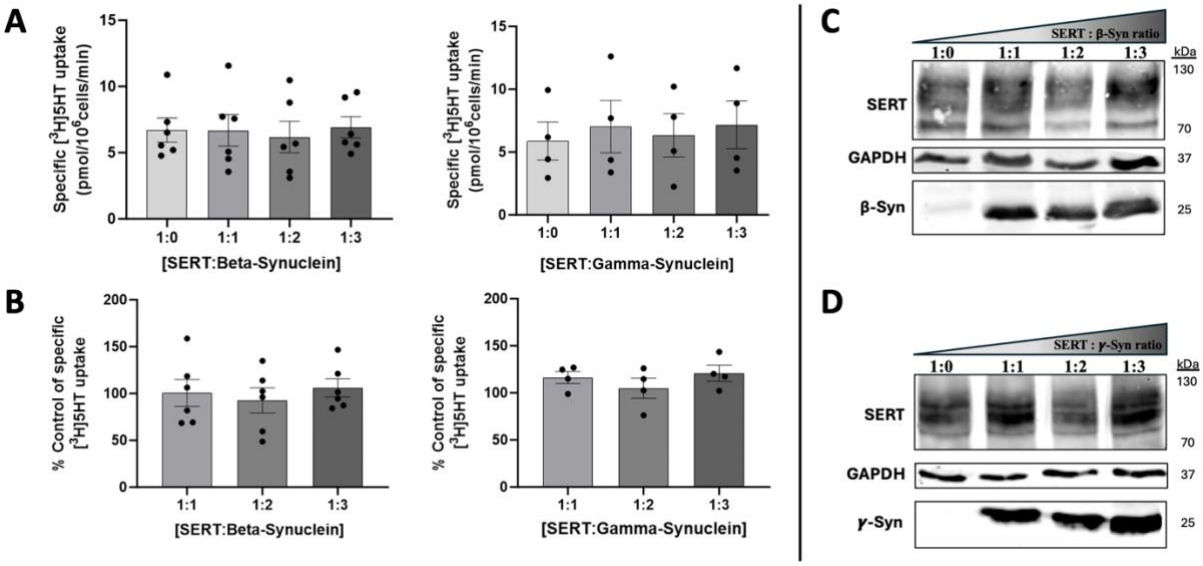
The influence of α-Syn on SERT activity and cell surface expression was previously explored in the Steinkellner lab (Sofic, 2024). However, the roles of β and γ-Syn in this context remain unexplored. These proteins may regulate important cellular processes either in cooperation with α-Syn or independently, potentially possessing distinct functions. Therefore, a key question is whether β-Syn and γ-Syn modulate MAT activity in a similar manner to α-Syn. The general aim of this thesis was to investigate the independent modulatory effects of all three synucleins on MATs. These aspects were studied under conditions of overexpression and gene silencing in cultured HEK-293 and Neuro2A (N2a) cells using biochemical and pharmacological methods. The specific aims were:

1. Examine the regulatory effects of β and γ-Syn on monoamine reuptake. Determine whether overexpression of β and γ-Syn alters MAT-mediated reuptake of monoamines through specific uptake assays.
2. Assess the cell surface expression of MATs and their colocalization with β-Syn or γ-Syn. Investigate the colocalization patterns of MATs with increasing concentrations of β-Syn or γ-Syn using immunocytochemistry and fluorescence microscopy. Determine expression levels of MATs via Western blotting.
3. Investigate the impact of α-Syn knockout on SERT activity. Generate *SNCA* -/- clones via CRISPR/Cas9-mediated gene editing. Confirm gene deletion via Western blotting and assess kinetic parameters of SERT-mediated reuptake of serotonin using uptake assays.
4. Use siRNA to reduce *SNCA* as an alternate gene silencing approach. Employ siRNA to knockdown *SNCA* expression. Confirm knockdown efficiency via Western blot and measure SERT activity using specific uptake assays.

2 Results

2.1 Impact of β-Syn and γ-Syn Overexpression on MAT Activity

The co-expressive effects of β-Syn or γ-Syn with MATs on monoamine reuptake were examined in the murine neuroblastoma cell line, N2a cells, using various transfection ratios. The specific reuptake of 5-HT into N2a cells increases when α-Syn is co-expressed with SERT at a ratio above 1:2 (SERT:α-Syn), as evidenced previously in the Steinkellner lab (Sofic, 2024). Based on this, similar monoamine uptake studies were performed to determine whether increasing levels of β-Syn or γ-Syn exhibit similar effects on MAT activity. N2a cells were co-transfected with a constant amount of SERT or DAT and increasing levels of β or γ-Syn (V5-tagged). Uptake of radiolabeled [^{3}H]5-HT and [^{3}H]DA was assessed under these conditions (**Figs. 2&3**).



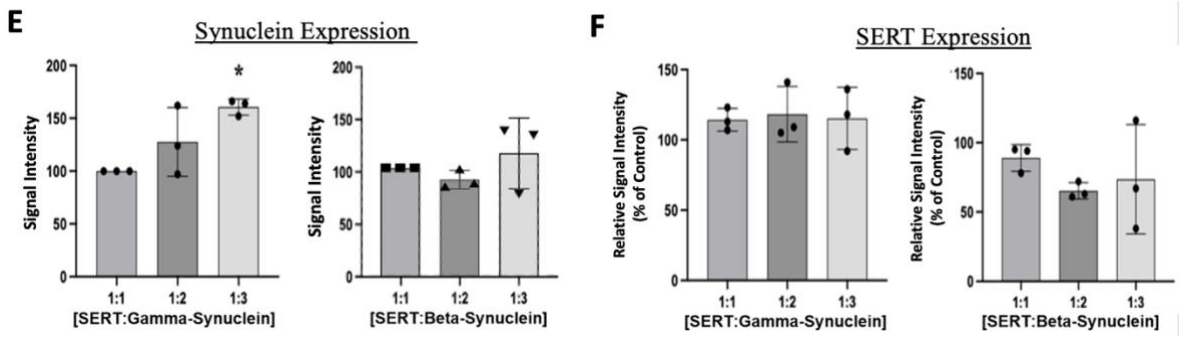


Figure 2: [^{3}H]5-HT uptake and DAT surface expression unaffected by overexpression of β-Syn or γ-Syn
Co-expression of β-Syn or γ-Syn with hSERT in N2a cells has no major effect on [^{3}H]5-HT uptake (100 nM) or hSERT expression. Nonspecific uptake was controlled using 10 μM paroxetine. Cells were co-transfected with 0.5 μg of hSERT and increasing amounts of synuclein (containing a V5 epitope tag) and/or with an 'empty vector' (pcDNA3.1) used to maintain constant total DNA amounts. **(A)** Absolute [^{3}H]5-HT uptake values (pmol/million cells/minute) for co-expression of hSERT and β-Syn (*Left graph*) and γ-Syn (*Right graph*). **(B)** Relative [^{3}H]5-HT uptake values, expressed as a fraction of the control condition (1:0 without synuclein), normalized to 100%.
Co-expression of hSERT and β-Syn *(Left graph*) and γ-Syn *(Right graph)*. Error bars represent standard error of the mean (SEM). Data shown as mean of the means ± SEM; β-Syn (n=6) and γ-Syn (n=4) from independent experiments performed in triplicate. Statistical significance was evaluated using one-way ANOVA followed by Dunnett's post hoc test, $P < 0.05$; (also *cf.* **Table 1**). Representative Western blot analysis for hSERT co-expressed with β-Syn **(C)** or γ-Syn **(D)**. eYFP-hSERT detected using polyclonal rabbit anti-GFP antibody (1:5000), and β/γ-Syn with monoclonal rabbit anti-V5 tag antibody (1:2000). No apparent change in of hSERT protein expression compared to untransfected control. Protein levels are the same for overexpressed β-Syn & γ-Syn (**C**&**D**; *Bottom blots*). Molecular size markers are shown in kilodaltons (kDa). **(E)** Densitometric analysis of β-Syn & γ-Syn overexpression, normalized to 1:1 transfection ratio (n=3 independent experiments). Statistical significance evaluated using one one-way ANOVA followed by Dunnett's post hoc test, $P < 0.05$. Significant difference between 1:1 & 1:3 SERT:γ-Syn transfection ratio, $^*P < 0.05$ (*Left graph)*. No significant differences between SERT:β-Syn transfection ratios (*Right graph*). **(F)** hSERT protein levels normalized to 1:0 untransfected control. Average protein levels quantified using ImageJ based on signal intensities from Western blots (*panels*: **C**&**D**). Protein expression levels normalized to GAPDH. No significant differences between increasing transfection ratios of SERT:Synuclein compared to 1:0 untransfected control (evaluated using one-way ANOVA followed by Dunnett's post hoc test, $P < 0.05$, n=3 independent experiments).

2.1.1 MAT Activity is Unaffected by Overexpression of β-Syn and γ-Syn

Relative to a defined control ratio of 1:0 (expressing only hSERT and an 'empty vector'), co-expression of β-Syn or γ-Syn with hSERT show no significant differences in specific [^{3}H]5-HT uptake across different transfection ratios (**Fig. 2A-B**). Similar to SERT, co-expression of β-Syn or γ-Syn with DAT did not significantly alter [^{3}H]DA uptake **(Fig. 3A&B).** Western blot analysis demonstrated no apparent changes in SERT and DAT protein expression levels, when co-expressed with increasing amounts of β-Syn or γ-Syn (**Fig. 2C&D, Fig. 3C&D**). This mirrors the lack of concentration-dependent β-Syn or γ-Syn induced changes in specific uptake activity described. hSERT and hDAT have a molecular weight of around 70-80 kD, but may appear higher due to the N-terminal enhanced yellow fluorescent protein (eYFP) tag and post-translational glycosylation (**Fig. 2C&D, Fig. 3C&D**). This range of molecular sizes for each specific isoform is consistent with other findings from Wersinger et al. (2003 & 2006). Mature glycosylated hDAT (90 kD) and immature forms (60 kD) have also been evidenced (Bjerggaard et al., 2004).

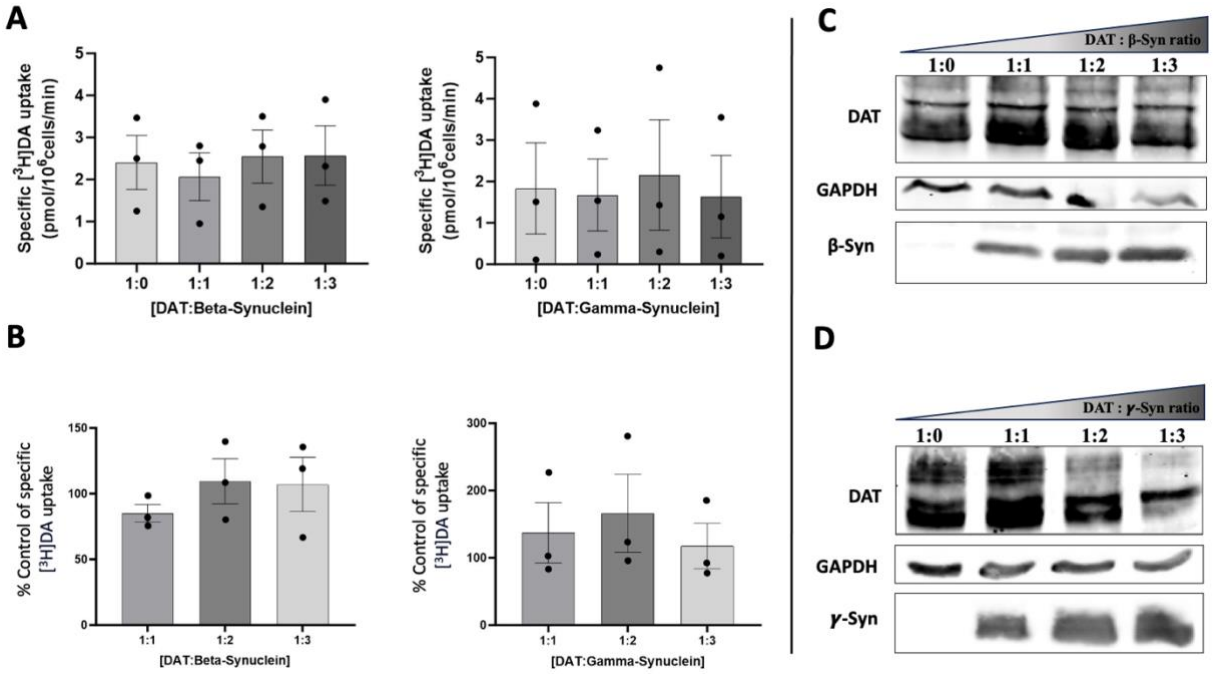


Figure 3: [^{3}H]DA uptake and DAT surface expression unaffected by overexpression of β-Syn or γ-Syn. N2a cells co-expressed with hDAT and increasing levels of β-Syn or γ-Syn. Experimental setup and statistical analysis were performed exactly as described in **Fig. 2**, substituted with hDAT and [^{3}H]DA (100 nM). Three independent experiments (n=3) for both β-Syn & γ-Syn specific uptake assays, performed in triplicate. Representative western blot for **(C)** β-Syn and **(D)** γ-Syn. n=1. Quantification of protein expression not shown.

Importantly, γ-Syn protein expression levels increased proportionally with their respective transfection ratios (**Fig. 2E)**, with significant differences in γ-Syn expression between 1:1 & 1:3 transfection ratios. β-Syn showed more variability in expression, with no significant differences determined by densitometric analysis. Quantification of signal intensities from Western blots further verified consistent SERT expression, relative to untransfected control, with no significant differences observed across overexpressed conditions (**Fig. 2F**). These results contrast with the previously observed effects of α-Syn and suggest that the overexpression of β-Syn and γ-Syn have no apparent effect on the rate of MAT-mediated specific uptake and are presumably not mediating a functional effect on the expression and/or uptake activities of SERT and DAT in N2a cells.**)**. A statistical summary for [^{3}H]5-HT and [^{3}H]DA uptake in N2a cells is shown in **Table 1**, indicating no significant changes for serotonin and dopamine reuptake in response to increasing expression levels of β-Syn or γ-Syn.

Comparison		Relative P Value	Absolute P Value	Sig. Diff. P < 0.05	Relative F Value	Absolute F Value	DF	n
hSERT : β-Syn	1:0 vs 1:1	>0.9999	>0.9999	ns	0.2516	0.0912	20	6
	1:0 vs 1:2	0.9331	0.9778	ns				
	1:0 vs 1:3	0.9619	0.9988	ns				
hSERT : γ-Syn	1:0 vs 1:1	0.3291	0.9436	ns	1.658	0.1095	12	4
	1:0 vs 1:2	0.9379	0.9958	ns				
	1:0 vs 1:3	0.1734	0.9236	ns				
hDAT : β-Syn	1:0 vs 1:1	0.7920	0.9637	ns	0.6270	0.1318	8	3
	1:0 vs 1:2	0.9296	0.9972	ns				
	1:0 vs 1:3	0.9670	0.9955	ns				
hDAT : γ-Syn	1:0 vs 1:1	0.8517	0.9991	ns	0.4908	0.0483	8	3
	1:0 vs 1:2	0.5483	0.9929	ns				
	1:0 vs 1:3	0.9793	0.9983	ns				

Table 1: No significant differences in monoamine specific uptake between transfection ratios.
Co-expression of increasing β-Syn or γ-Syn with either hSERT or hDAT. Summary of statistical significance between MAT:Synuclein transfection ratios and 1:0 untransfected control evaluated using one-way ANOVA followed by Dunnett's pos-hoc analysis for multiple comparisons, $P < 0.05$. n=number of independent experiments performed in triplicate.

2.1.2 Synucleins Colocalize with hMATs Near the Plasma Membrane

A previous study demonstrated that increasing SERT:α-Syn transfection ratios of synuclein significantly elevated the cell surface expression of SERT, showing colocalization with α-Syn at or near the plasma membrane (Sofic, 2024). To explore whether β-Syn or γ-Syn associate similarly with SERT or DAT in N2a cells, immunocytochemical analysis was performed to visualize their subcellular localization. Since N2a cells do not express hSERT and hDAT endogenously (based on radioligand uptake studies performed in Sofic, 2024), the respective N-terminally tagged eYFP-hMAT was transfected throughout experiments. The subcellular location of hSERT and hDAT along with β-Syn or γ-Syn (using the V5 antibody) were visualized by fluorescence microscopy.

The analysis revealed substantial colocalization of the synucleins with the transporters at various transfection ratios (**Fig. 4**). Primary antibodies against hSERT and hDAT were used in conjunction with anti-V5 antibodies to detect V5-tagged β-Syn and γ-Syn. Cells expressing MATs in the absence of synuclein (1:0 condition) serve as an experimental control. The immunoreactivity of the synuclein with their respective MAT is detectable throughout the cell, including the cytosol and at the cell membrane. (**Fig. 4**).

As expected, the fluorescence intensity of SERT and DAT was higher at the cell membrane where it normally localizes (**Fig. 4A-D**, *YFP-SERT & DAT columns*). Merged images suggested colocalization of synuclein and MAT at or near the plasma membrane (**Fig. 4A-D,** *Overlay columns*). The distribution of SERT appears evenly spread out with no apparent changes in density at the membrane surface (**Fig. 4E**).

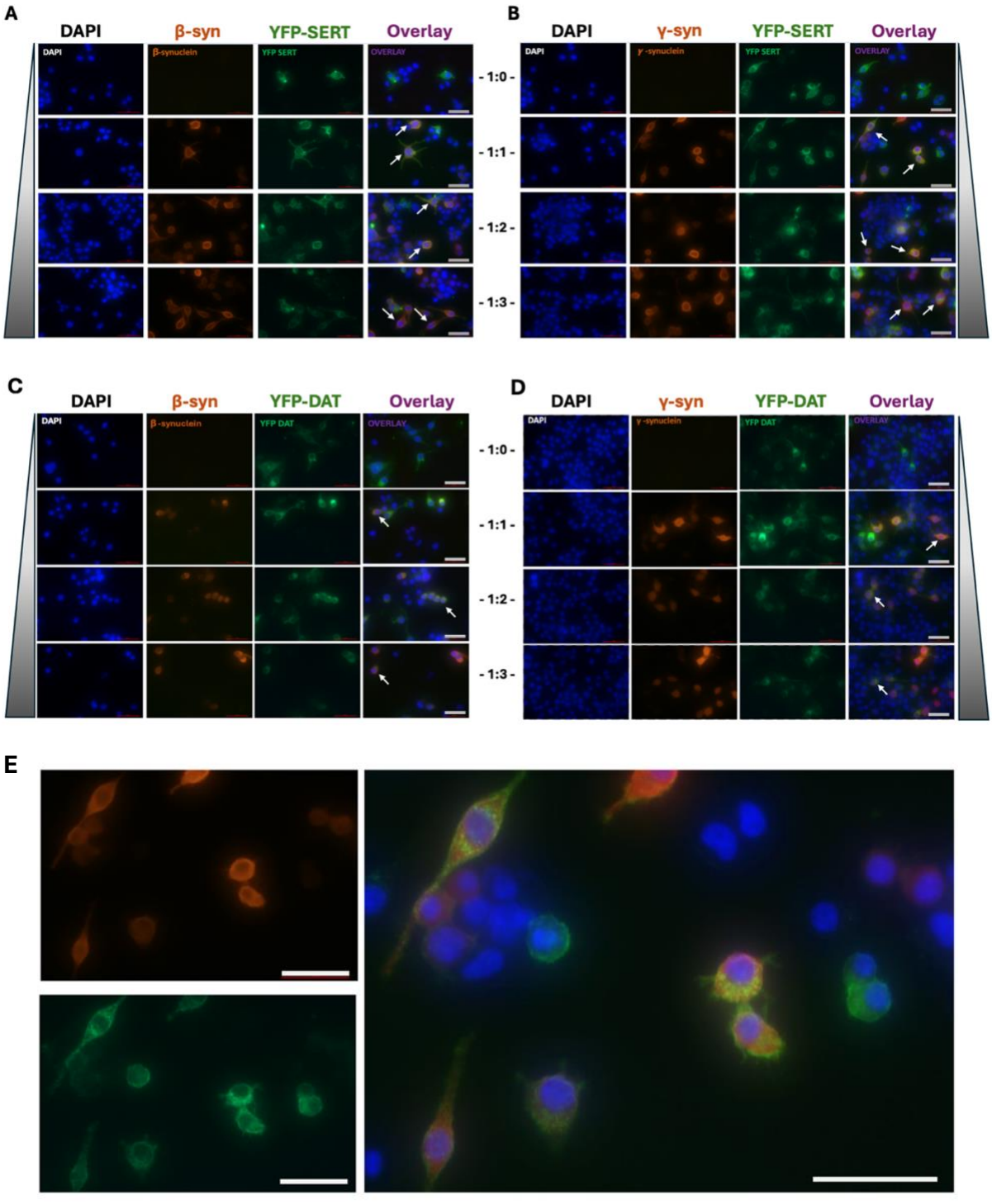


Figure 4: Immunocytochemistry reveals colocalization of β or γ-Syn with hSERT/DAT in N2a cells.
Co-expression of YFP-hSERT with increasing amounts of **(A)** β-Syn or **(B)** γ-Syn. Co-expression of YFP-hDAT with increasing amounts of **(C)** β-Syn or **(D)** γ-Syn. YFP-hSERT/hDAT detected using primary monoclonal mouse anti-SERT or rat anti-DAT antibody (1:500) & β/γ-Syn monoclonal rabbit anti-V5 tag antibody (1:1000). Secondary antibodies for SERT detected using anti-mouse AlexaFluor488 or anti-rat AlexaFluor594 (both 1:1000) and goat-anti rabbit AlexaFluor568 (1:1000). Selected fluorescence images display immunoreactivity distribution of DAPI (blue), synuclein (red), SERT/DAT (green), and co-localization (overlays - arrows highlight co-localization sites). Expression level of β-Syn or γ-Syn is undetectable by V5 antibodies in untransfected control (1:0). Cells imaged by fluorescence microscope and selected from a collection of several fields of view. Digital images taken at 40x-scalebar:50 µm and are representative of various independent experiments; β-Syn:hSERT (n=6), γ-Syn:hSERT (n=4), β/γ-Syn:hDAT (n=3). **(E)** Representative image of YFP-hSERT:γ-Syn (1:1 ratio), scaled-up for improved visual of cellular distribution and colocalization.

Fig. 5 confirms co-transfection by surveying approximately 200 cells, identifying SERT positive (green), synuclein positive (red), and double-positive cells (overlay of both proteins). These images provide a broader view of cells co-expressing MATs with β-Syn or γ-Syn. Colocalization was quantified as the percentage of SERT-positive cells that were also positive for synuclein. Approximately 90% of the SERT-positive cells were double positive, indicating a pattern of co-localization between SERT and either β-Syn or γ-Syn at the plasma membrane.

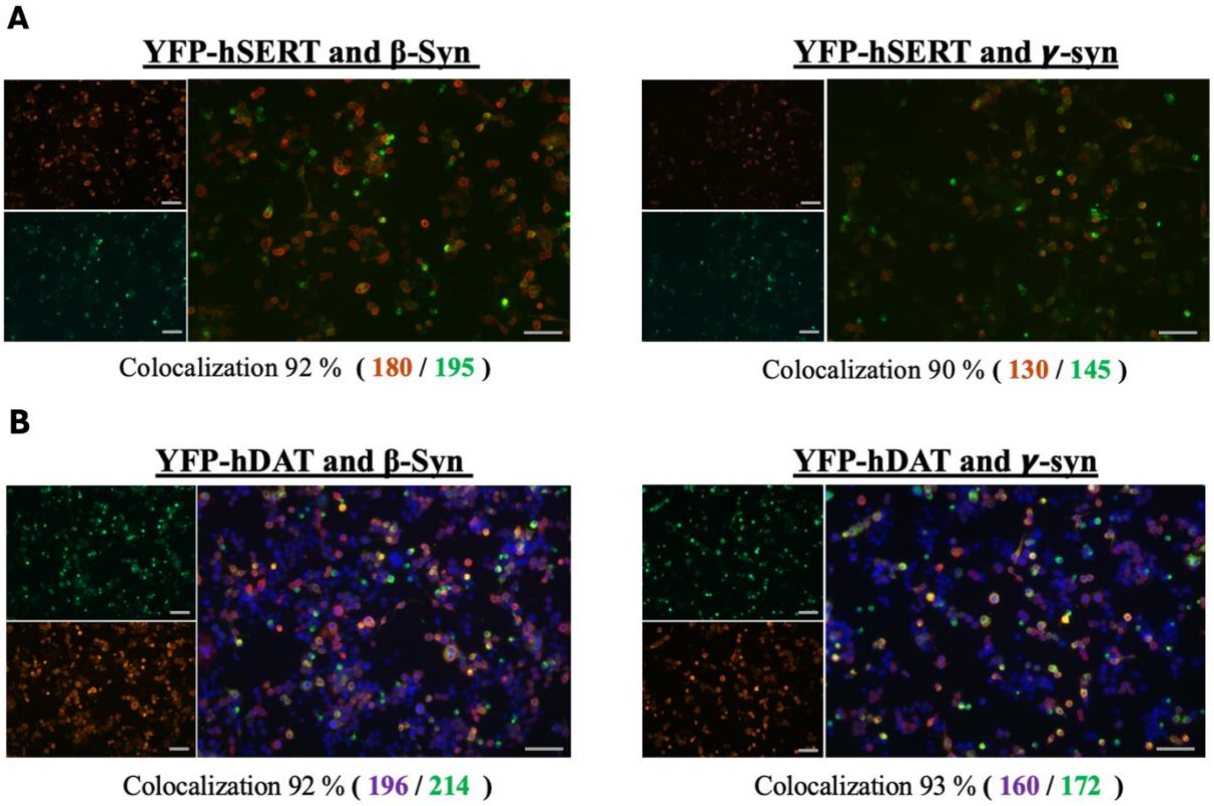


Figure 5: Colocalization rates between hSERT and hDAT with either β-Syn or γ-Syn.
Representative immunocytochemical analysis of β-Syn or γ-Syn co-transfected with hSERT **(A)** and hDAT **(B)** in N2a cells at 1:3 ratio. Colocalization was visualized as an overlay of the indicated proteins, with SERT/DAT-positive (green) and β or γ-Syn (red). Colocalization was quantified as the percentage of SERT-positive cells that also expressed β or γ-Syn. The images were captured using a fluorescence microscope at 10x-scalebar:100 µm and represent several digital images collected from four independent experiments (n=4).

Colocalization was further evaluated by immunofluorescence in cells co-transfected with either β-Syn or γ-Syn alongside eYFP-SERT **(Fig. 6A)** or eYFP-DAT **(Fig. 6B)**. Despite this colocalization, immunocytochemical analysis showed that neither β-Syn nor γ-Syn affected the distribution density or localization intensity of SERT or DAT at the membrane, as indicated by unchanged fluorescence intensity profiles **(Fig. 6)**.

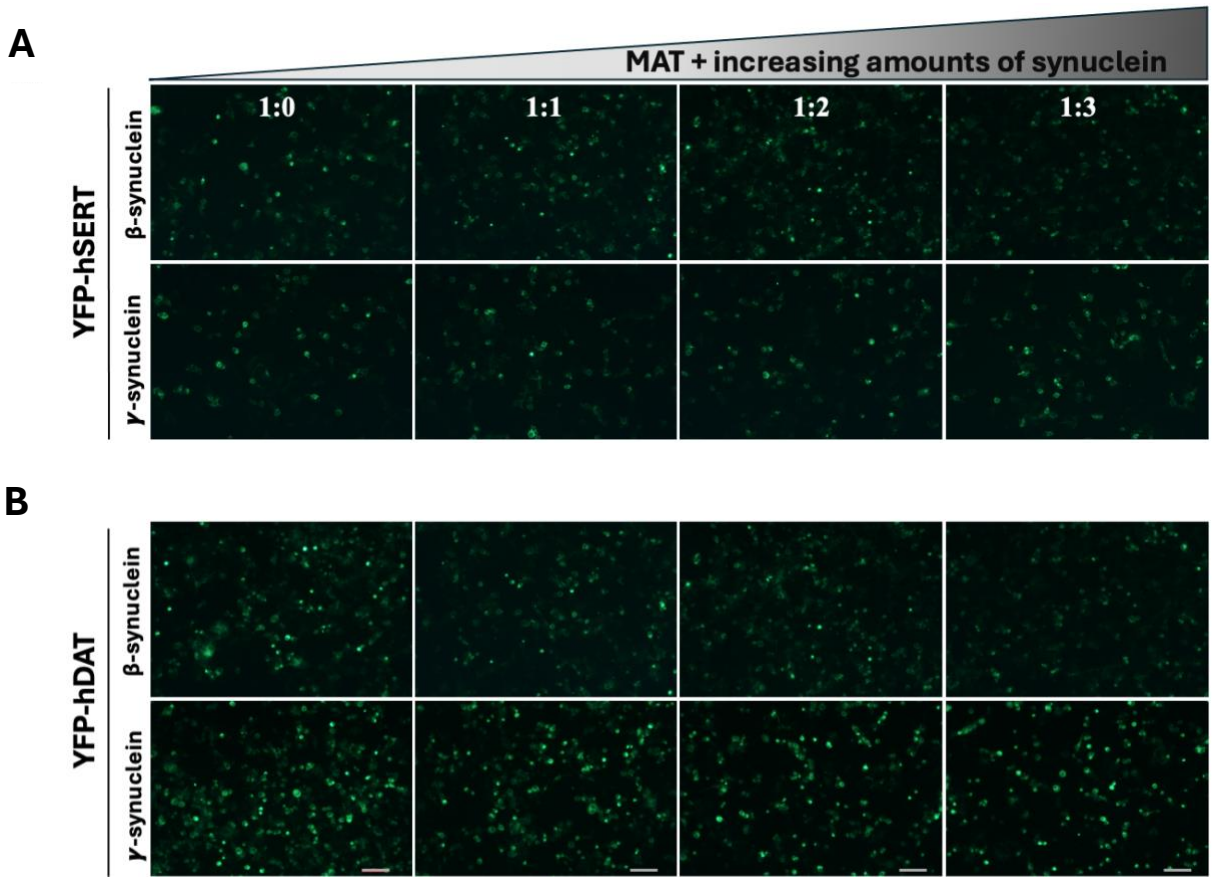


Figure 6: Unchanged immunofluorescence intensity of YFP-hSERT/hDAT when co-expressed with β/γ-Syn. **(A)** eYFP-SERT and **(B)** eYFP-DAT; co-transfected with increasing amounts of either β-Syn or γ-Syn. The immunoreactivity of SERT and DAT was visualized using an anti-GFP (+ Alexa Flour 488 (green)) targeted against eYFP tag on hSERT/DAT (1:1000). Digital immunofluorescence images captured using a fluorescence microscope at 10x-scalebar:100 µm and represent signal intensities from two different fields of view. These images are derived from four independent experiments.

2.2 Effects of RNAi-mediated α-Syn Knockdown on Serotonin Uptake in N2a and HEK293 Cells

Several siRNAs targeting endogenous *SNCA* transcripts (predesigned by Invitrogen, USA), were screened for their ability to achieve RNAi-mediated knockdown of *SNCA* in HEK293 and N2a cells. Initially, murine siRNA03 targeting regions of the mouse *SNCA* mRNA (encoding α-Syn protein) was tested for its efficacy in N2a cells, but experiments were discontinued because we detected only minimal levels of endogenous α-Syn expression in N2a cells (Sofic, 2024).

Our focus shifted to HEK293 cells, which is a human cell line. Unexpectedly, endogenous *SNCA* expression is quite abundant in these cells, as demonstrated by several other students in the Steinkellner lab (*also see* **Fig. 8B**). Hence, human specific siRNAs were used to knockdown endogenous α-Syn in HEK293 cells for subsequent experiments.

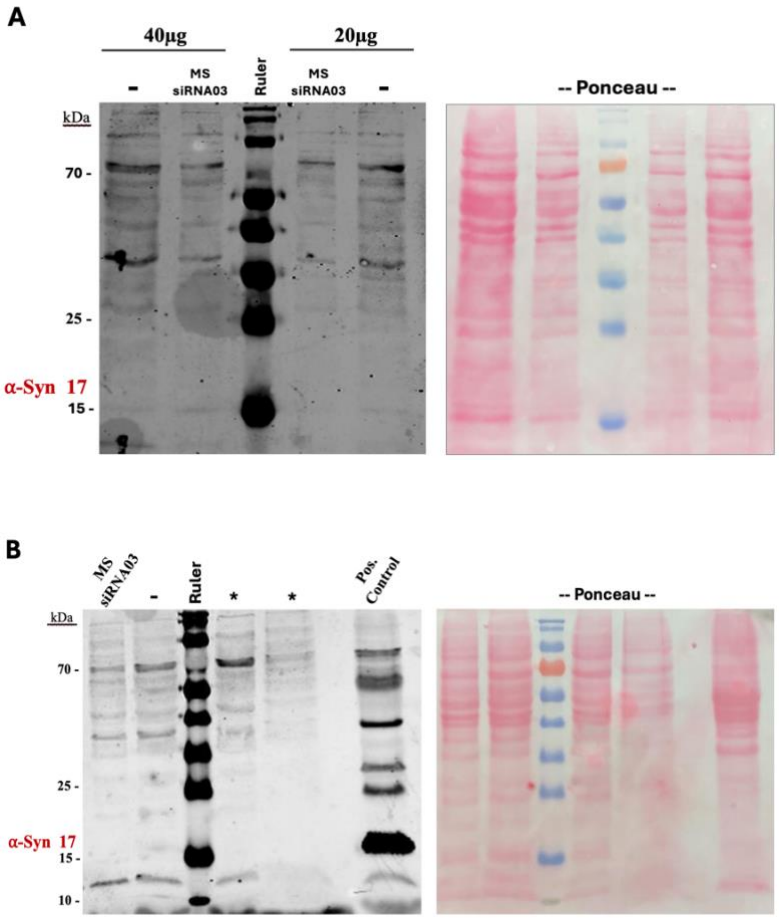


Figure 7: Silencing of endogenous *SNCA* using a predesigned murine siRNA in N2a cells.
N2a cells co-transfected with hSERT and murine siRNA03 (10 nM) targeted against murine *SNCA*. **(A)** Western blot loading screen using 20 µg and 40 µg of protein. α-Syn expression detected using α-Syn monoclonal antibody 4D6 (1:1000), against endogenous α-Syn. Ponceau S staining as loading control. *Alternative experiment and should be neglected.

(B) Representative Western blot from cells treated with siRNA03; α-Syn detected using α-Syn monoclonal antibody 4D6 (1:1000). Mouse protein lysate from striatum used as α-Syn positive control. Ponceau S staining as loading control. n=3.

2.2.1 siRNAs Reduce Serotonin Uptake in HEK293 Cells

Serotonin uptake assays were conducted in HEK293 cells transiently co-transfected with eYFP-hSERT and each of the siRNA constructs **(Fig. 8)**. For these experiments, cells from both the negative control siRNA and siRNAs targeting human *SNCA* were analyzed together. one-way ANOVA indicated no significant differences when comparing each siRNA treatment to the negative control (**Fig. 8A)**.

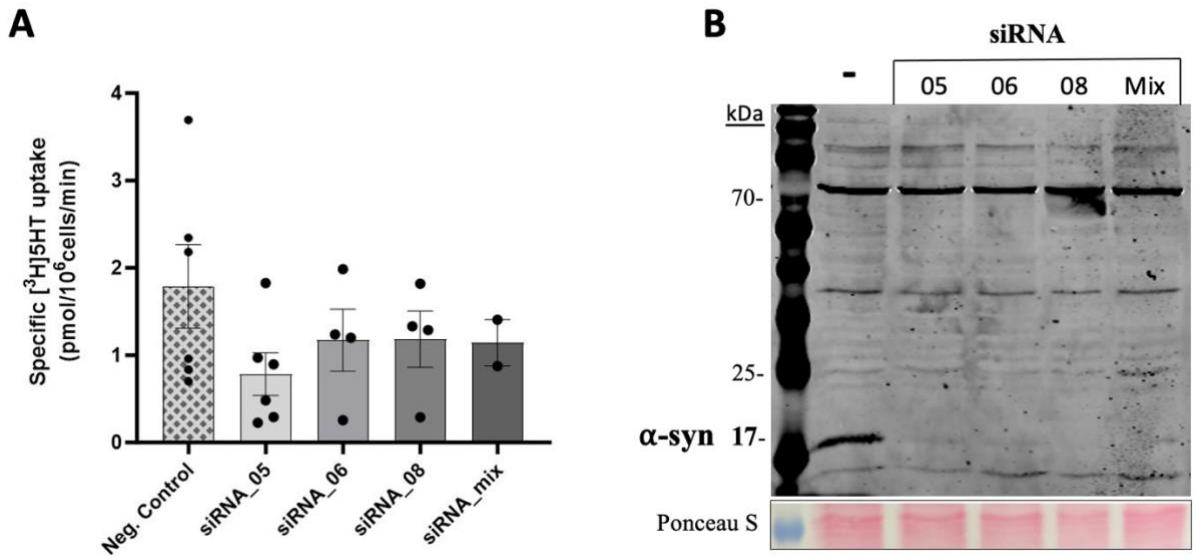


Figure 8: Three different human siRNAs reduce endogenous α-Syn expression levels. HEK293 cells transfected with eYFP-hSERT and three siRNAs (10 nM) of different target sequences (referred to as siRNA05,06&08, plus one mixed sample). **(A)** Combined assessments of [^{3}H]5-HT reuptake (0.1 µM) for cells treated independently with either of the siRNAs and negative control siRNA. Absolute values expressed as mean of the means ± SEM. No significant differences between siRNA treatments compared to negative control (one-way ANOVA followed by Dunnett's multiple comparisons test, $P < 0.05$). Multiple independent experiments siRNA05 (n=6), 06 (n=4), 08 (n=4) and mix (n=2), performed in triplicate. **(B)** Representative Western blot validating moderate *SNCA* knockdown detected by mouse α-Syn monoclonal antibody 4D6 (1:1000) against endogenous human α-Syn. Ponceau S staining as loading control (25 kD). n=3.

Although the *SNCA* knockdown experiments were successful, overall reduction in serotonin uptake varied across siRNA treatments. siRNA05 was able to decrease the serotonin uptake by 65% compared to negative control (**Fig. 8A**), while the impact of siRNA 06 and 08 on serotonin uptake was less pronounced (20 - 30% reduction). Western blot analysis qualitatively confirmed reduction in α-Syn expression levels in all three siRNA treated samples, with (almost) no detectable bands observed **(Fig. 8B).** As expected, neither the *SNCA* nor α-Syn expression levels were affected by negative control siRNA. Notably, SERT and α-Syn expression levels were not normalized to GAPDH loading controls, limiting quantitative analysis. Although qualitative changes in SERT activity were observed through serotonin uptake assays, additional

experiments are required to assess changes in SERT protein levels. Still, the data suggest there is a change in SERT function due to substantial silencing of α-Syn, revealing all three siRNAs can reduce endogenous expression of *SNCA* to some extent.

2.2.2 RNAi-mediated Knockdown of α-Syn Affects SERT Localization

The intracellular distribution and cell surface expression of YFP-tagged SERT were evaluated via fluorescence microscopy. Under normal conditions, SERT is concentrated at the plasma membrane when co-expressed with α-Syn (Sofic, 2024). RNAi-mediated knockdown of α-Syn appeared to modestly affect YFP-SERT expression and cell surface localization, as observed 72 hours post-transfection **(Fig. 9)**. Fluorescence intensity of YFP-SERT was dimmer in siRNA05 treated cells, with fewer fluorescent signals compared to the brighter control group. These observations suggest potential alterations in SERT cell surface expression and distribution patterns. To ensure accurate interpretation of fluorescence signals, differences in cell viability were considered by normalizing cell counts to the negative control. The cell counts were notably reduced by siRNA treatments, suggesting that the observed reduction in fluorescence might be partially due to decreased cell viability Although these visual observations are preliminary and qualitative, they align with the reduced serotonin uptake observed in **(Fig. 8)**, suggesting a functional interaction between α-Syn and SERT.

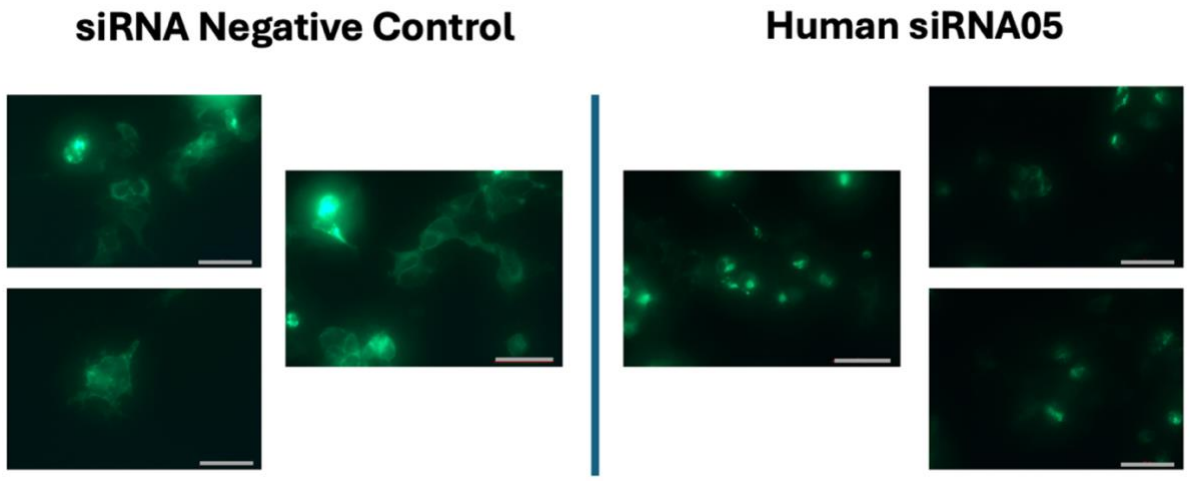


Figure 9: siRNA05 mediated knockdown effect on eYFP-hSERT expression & cell viability in HEK293 cells. Digital images detect green fluorescence signal of endogenous eYFP tagged SERT at the N-termini. Representative images collected from two different fields-of-view (n=6). Taken 72 hours after primary siRNA transfection by fluorescence microscope at 40x-scalebar:100 µm.

2.3 CRISPR-mediated Deletion of *SNCA* and Effects on Serotonin Reuptake in N2a and HEK93 Cells

2.3.1 SNCA Knockout in N2a cells

The role of α-Syn in modulating SERT activity was evaluated by disrupting *SNCA* in HEK293 and N2a cell lines using clustered regularly interspaced short palindromic repeats CRISPR-associated protein 9 (CRISPR-Cas9)-mediated gene editing. Initial attempts to eliminate the endogenous α-Syn expression in N2a cells produced unclear results via Western blot analysis, presumably related to the low endogenous expression of α-Syn in N2a cells. Serotonin specific uptake assays at a single-concentration (0.1 µM [^{3}H]5-HT), yielded inconsistent data, and was incomparable to WT controls **(Fig. 10A)**. Minimal reductions in *SNCA* expression were observed in the presumed knockout clones (19* & 23*) compared to an assumed WT cognate (7* & 11*), as confirmed by Western blot analysis (**Fig. 10B**). This result is likely due to low endogenous expression of α-Syn in N2a cells.

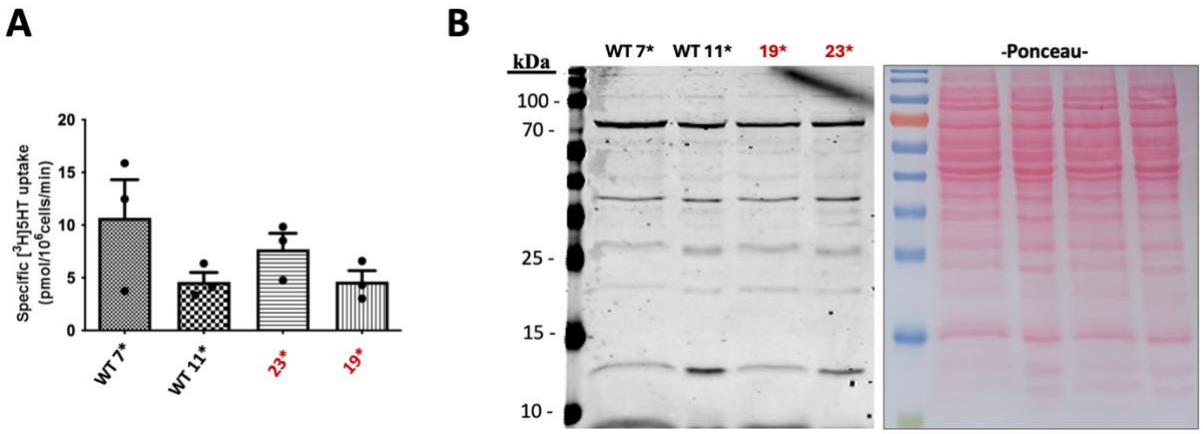


Figure 10: Assessment of CRISPR-Cas9-mediated knockout of *SNCA* in N2a cells.
(A) Absolute serotonin uptake values (0.1 µM [^{3}H]5-HT), expressed as mean of the means ± SEM. No significant differences observed when compared to putative WT controls (one-way ANOVA, $P < 0.05$, n=3). **(B)** Representative Western blot of two putative SNCA $^{-/-}$ clones (19* & 23*), plus supposed WT monoclonal cells (7* & 11*). α-Syn protein expression levels detected using mouse α-Syn monoclonal antibody 4D6 (1:1000). n=3.

Additionally, a T7 endonuclease assay (T7E1) did not indicate any DNA mismatches in the targeted *SNCA* region, suggesting that CRISPR-Cas9 editing was either inefficient or incomplete (data not shown). The region surrounding the presumed *SNCA* mutation was amplified by Polymerase chain reaction (PCR). No clear evidence of gene disruption was observed. The putative loss of *SNCA* mRNA was also tested by qPCR using primers specific to *SNCA* mRNA listed in **Materials and Methods**. Briefly, RNA was isolated from single-cell clones for screening *SNCA* knockout from synthesized cDNA. However, levels of *SNCA*

transcripts in the knockout clones were undetectable, as indicated by the absence of ct values (data not shown). This could be explained by the characteristic low levels of *SNCA* mRNA in N2a cells, making it difficult to accurately quantify the loss of *SNCA* expression.

2.3.2 SNCA Knockout in HEK293 Cells

Given the challenges in N2a cells, a second attempt of CRISPR-Cas9 mediated knockout experiments was performed in HEK293 cell. The expression of endogenous alpha synuclein was previously shown to be more abundant in HEK293 cells (*see above*). Therefore, a new *SNCA* sgRNA targeting exon 4 of human *SNCA* was purchased (Integrated DNA Technologies, Iowa) and transfected into HEK293 cells. Six candidates (single cell *SNCA* knockout clones) were expanded and screened via Western blot (**Fig. 11**) Four clones with an absence of α-Syn protein expression were identified as potential knockouts (# 24, 29, 35, and 51) while two cognate clones exhibited no visible reduction in band intensity and were designated as WT controls (# 49 & 53) **(Fig. 11B).** Furthermore, *SNCA* $^{-/-}$ clone (#24) exhibited the most prominent reduction in band intensity and was selected for propagation into a stable cell line.

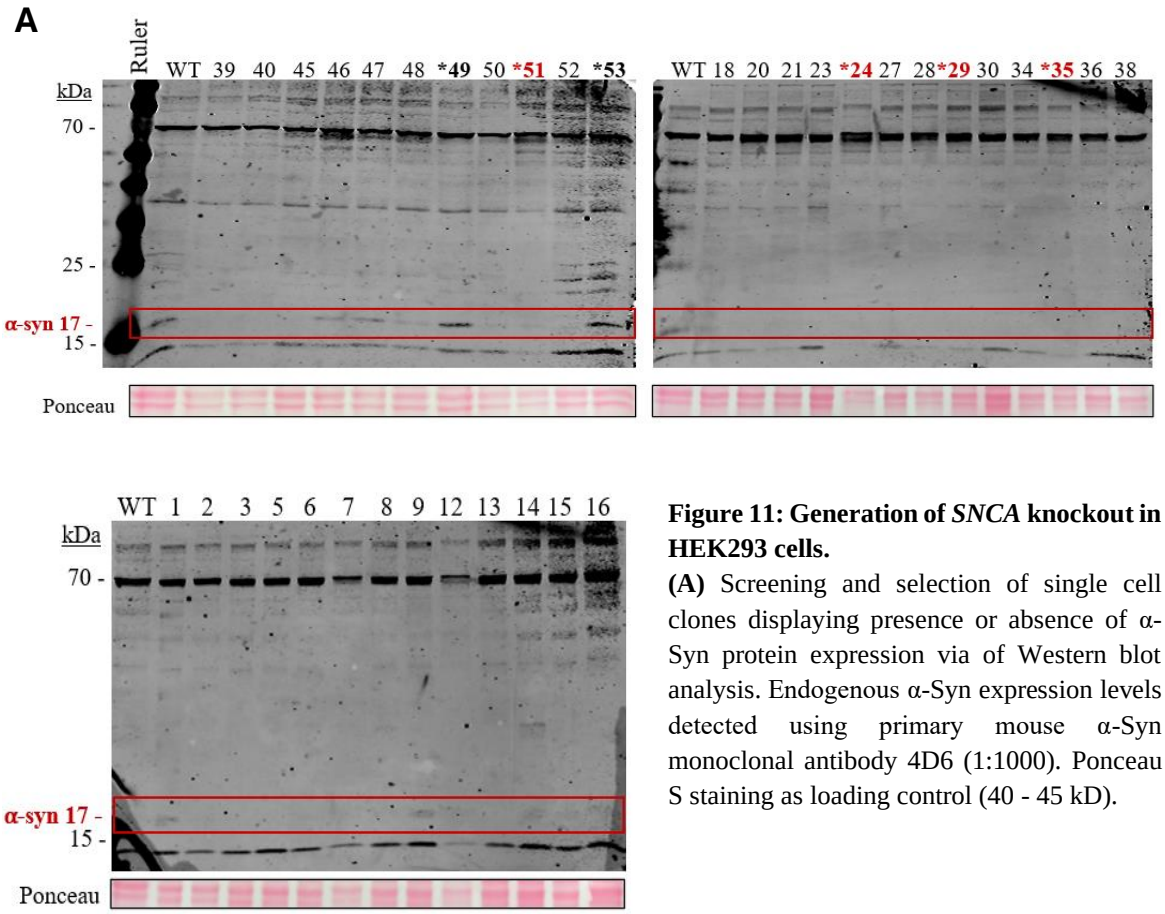


Figure 11: Generation of *SNCA* knockout in HEK293 cells.
(A) Screening and selection of single cell clones displaying presence or absence of α-Syn protein expression via of Western blot analysis. Endogenous α-Syn expression levels detected using primary mouse α-Syn monoclonal antibody 4D6 (1:1000). Ponceau S staining as loading control (40 - 45 kD).

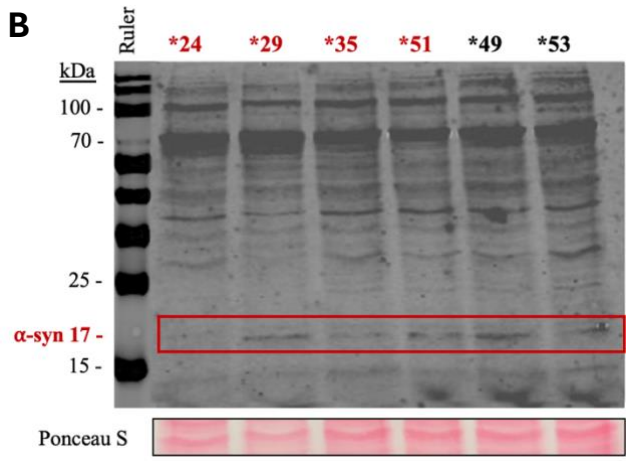


Figure 11: (continued)
(B) Western blot confirmation of candidate cells expressing non-target cognate *SNCA* clones (WT control Black*), plus four *SNCA* knockout clones (Red*). Endogenous α-Syn expression levels detected using primary mouse α-Syn monoclonal antibody 4D6 (1:1000). Ponceau S staining as loading control (37 kD).

2.3.3 Cell Viability and Morphology of SNCA Knockout Clones

The selected *SNCA* $^{-/-}$ clone (#24) displayed a similar morphology as cognate WT clone (#49), as observed through fluorescence microscopy at twenty-four and forty-eight hours post transfection with SERT **(Fig. 12)**. The cell proliferation rate of *SNCA* $^{-/-}$ clone (#24) was visually unaffected by the absence of *SNCA*, and this clone was deemed suitable for generating a stable HEK293 *SNCA* knockout cell line confirmed by Western blot. This cell line (referred to as *SNCA* -/- HEK293 cells from now on) were subsequently employed to further investigate the modulatory effect of α-Syn on serotonin uptake.

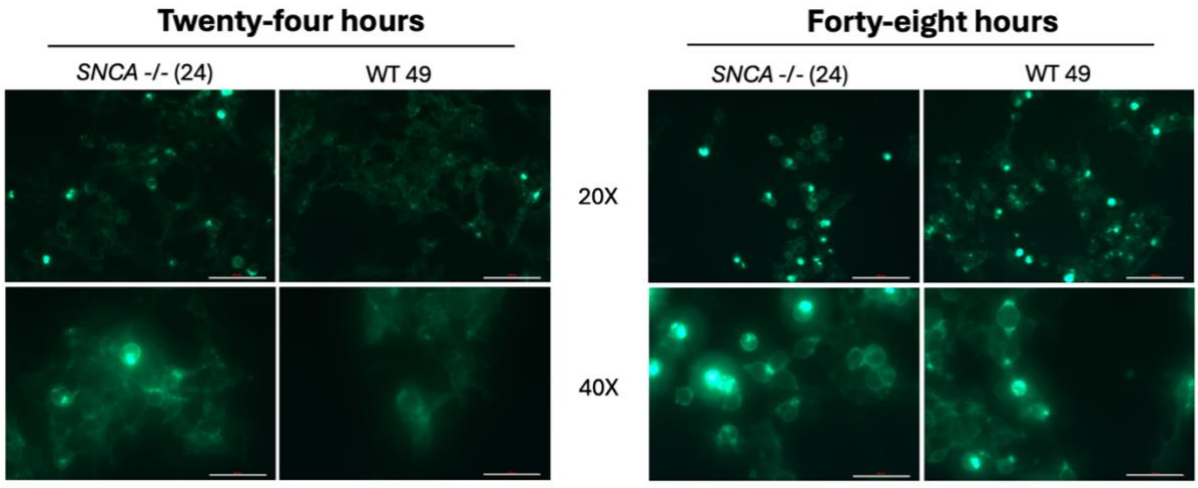


Figure 12: Morphology & endogenous YFP-SERT expression of the prospective HEK293 *SNCA* -/- cell line. Cells were transfected with hSERT using Lipofectamine 2000 and then live imaged by fluorescence microscope at 24 and 48-hour time periods. Representative images of cellular viability of the SNCA -/- cells (# 24) compared to cognate WT clone (# 49). Scalebar: 50 µm (40x), 100 µm (20x). n=3.

2.3.4 SNCA Knockout Reduces Serotonin Uptake in HEK293 Cells

Kinetic analyses were conducted to assess the effect of α-Syn knockout on serotonin uptake in *SNCA* -/- HEK293 cells (K.O #24) transfected with hSERT. The kinetic constants, V_{max} and K_m were derived by fitting a Michaelis-Menten model and using an extra-sum-of-squares F test ($P < 0.05$) for comparing variance between *SNCA* -/- HEK293 cells and a cognate WT (WT 49) control (**Fig. 13A**). The V_{max} of *SNCA* -/- HEK293 cells (47.49 ± 9.46 pmol/millioncells/min) was significantly reduced compared to WT control (98.49 ± 14.50 pmol/millioncells/min). However, the K_m values between *SNCA* -/- HEK293 cells and WT control (respectively, 0.98 µM ± 0.99 µM *versus* 1.80 µM ± 1.16 µM) were not significantly different when using the same extra sum-of-squares F test for comparison ($P < 0.05$). Given the significant reduction in V_{max} but not K_m, in *SNCA* -/- HEK293 cells compared to WT cells, the affinity of SERT for serotonin was unaffected by the loss of *SNCA*. α-Syn appears to modulate V_{max} but not the binding affinity of SERT.

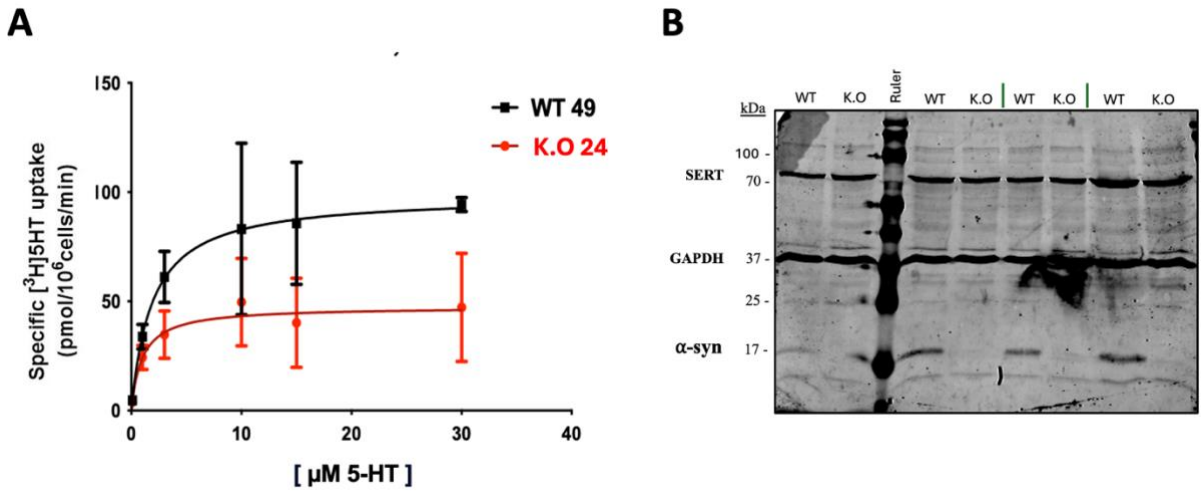


Figure 13: α-Syn mediates reduction of SERT activity in *SNCA* $^{-/-}$ HEK293 cells.
(A) Representative kinetic analysis of *SNCA* knockout effects in HEK293 cells transfected with YFP-hSERT Serotonin uptake velocity of *SNCA* $^{-/-}$ cells (K.O. #24) is significantly different than WT control (Extra sum-of-squares F-test); d=27; F value=5.6, P value= 0.02, (*P < 0.05). Kinetic constants derived from Michaelis-Menten least squares nonlinear regression modeling. Best-fit values are expressed as mean of the means ± SEM performed in triplicate from three independent experiments (n=3). **(B)** Representative Western blot confirming absence of α-Syn expression compared to WT control from 4 independent experiments (n=4). Lysates boiled at 95 for 5 minutes and loaded onto SDS-Page in equal amounts. α-Syn protein detected using primary mouse α-Syn monoclonal antibody 4D6 (1:1000). GAPDH as loading control.

2.3.5 Confirmation of SNCA Knockout & Correlation with Uptake Activity

Western blot analysis was performed two days post transfection and confirmed a reduction in α-Syn expression levels for *SNCA* -/- HEK293 cells compared to WT (**Fig. 13B**). Minimal amounts of residual α-Syn protein were detected by densitometric analysis, likely reflecting background noise**.** The reduction in *SNCA* levels correlates with a decreased capacity for serotonin uptake **(Fig. 13A**), potentially due to reduced SERT density at the plasma membrane. Due to time constraints, quantitative evaluation of SERT protein expression in *SNCA* -/- HEK293 was not conducted in this study. Further analysis will be necessary to confirm whether the reduction in serotonin uptake observed in *SNCA* -/- HEK293 cells is a result of changes in SERT cell membrane expression.

3 Discussion

3.1.1 Role of Synucleins in Modulation of MAT Activity

The regulation of MAT activity (particularly SERT and DAT) by α-Syn, β-Syn and γ-Syn has been an ongoing focus in the synuclein field. However, effects of synucleins on MATs have varied considerably across studies. Some report that α-Syn overexpression decreases cell surface expression of MATs (Wersinger et al., 2003), while others observe increased surface expression (Lee et al., 2001). Previous work in the Steinkellner lab indicated that α-Syn overexpression increases the cell surface expression of SERT, amplifying serotonin reuptake in an α-Syn-dependent manner (Sofic, 2024). These discrepancies likely arise from differences in experimental systems, the intended synuclein expression levels, and methodologies, which may affect the availability of MATs at the cell surface. Due to these variations, the interaction between synucleins and MATs was reassessed in this study, under conditions of overexpression and gene silencing in neuronal and non-neuronal like cells, primarily focusing on SERT.

While all three synucleins may interact with and influence MAT activity, α-Syn is widely accepted as an important modulator of monoamine transporters with potential implications for Parkinson's disease (Oaks and Sidhu, 2011). Synucleins have known affinity for highly curved membranes and α-Syn is involved in synaptic vesicle (SV) dynamics by facilitating both endocytosis and exocytosis (Bendor et al., 2013 Middleton and Rhoades, 2010). These functions suggest that synucleins play a key role in regulation of synaptic transmission and are localized at presynaptic sites. In addition to their role in the SV cycle, synucleins influence MAT activity by modulating the trafficking of transporters to and from the presynaptic membrane (Oaks and Sidhu, 2011).

Although the roles of β-Syn and γ-Syn are less well-defined than those of α-Syn, they may cooperate with or function independently of α-Syn. My findings indicate that β-Syn and γ-Syn have no apparent effect on MAT activity when overexpressed. This aligns with research suggesting that β-Syn and γ-Syn may influence DAT trafficking at early stages of export from the endoplasmic reticulum (Oaks et al., 2013), but these effects do not extend to a direct modulation of surface expression levels, as observed in my experiments. The absence of significant effects on MAT-mediated reuptake after β-Syn and γ-Syn overexpression suggests that these synucleins do not influence the cell surface expression of MATs. One possible explanation is that unlike the aggregation-prone α-Syn, β-Syn is missing most of its NAC

region, which may limit essential interactions with MATs and affect their distribution at the cell surface. This may also explain β-Syn's reduced propensity to aggregate and form amyloid fibrils (Williams et al., 2018; Goedert, 2001). In fact, β-Syn acts as a molecular chaperone and has been shown to prevent α-Syn aggregation, possibly through direct binding to α-Syn (Lee et al., 2004; Hashimoto et al., 2001) Similarly, γ-Syn exhibits chaperoning activity and has been implicated in protein trafficking (Liu et al., 2007). Therefore, it is plausible that β-Syn and γ-Syn are tethered to chaperone-like roles rather than directly regulating MAT activity at the cell membrane.

The conformational dynamics of synucleins, ranging from unfolded monomers to aggregated amyloid structures, exist on a spectrum of possibilities (Gómez-Benito et al., 2020). The presence of these different forms (monomers, oligomers, or tetramers) in vitro could be determined using intact cell cross-linking protocols, as demonstrated by Dettmer et al. (2013) and Imberdis et al. (2019). Throughout this thesis, careful attention was given to accurately represent the synuclein monomer expression levels and avoid misinterpretation of protein structure, as assessed by Western blot analysis. The denaturing conditions of Western blotting may have affected synuclein detection, but this study aimed to preserve the synuclein's native structures and avoid overestimating aggregate formation. Synucleins primarily exist as monomers in vitro (Jain et al., 2018) and exhibit a pattern of folding and unfolding to exist in equilibrium (Bourdenx et al., 2017). Tetrameric forms of α-Syn are suggested to correspond with bands at approximately 55 kD (Bartels et al., 2011). Similarly, β-Syn has been shown to form tetramers (60 kD), though these structures destabilize into monomers due to lysis sensitivity (Dettmer et al., 2013). Since monomers can easily detach from membranes, applying paraformaldehyde was demonstrated to prevent the detachment of α-Syn from the membrane (Lee and Kamitani, 2011). To improve the detection of monomeric synuclein in this study, paraformaldehyde fixation was applied to the nitrocellulose membrane, as a part of the Western blotting methodology.

3.1.2 Synucleins and MATs are Co-expressed in the Same Cells

In addition to specific uptake assessments, immunocytochemistry (ICC) confirmed that β-Syn and γ-Syn independently colocalize with both DAT and SERT, consistent with previous findings for α-Syn colocalization with SERT (Sofic, 2024). However, unlike α-Syn, which increased SERT surface expression when overexpressed, the cell surface expression of SERT and DAT, remained relatively stable when co-expressed with β or γ-Syn, even across different

transfection ratios. This observation aligns well with the specific monoamine uptake assessments, which also showed no significant effects on MAT activity by β or γ-Syn.

Counting SERT positive and synuclein positive cells, was integral to confirming co-transfection. The results indicated that cells that incorporated β-Syn or γ-Syn do not seem to have a greater likelihood for expressing higher levels of MATs at the cell surface. More than 90% of the co-transfected N2a cells showed colocalization of either β-Syn or γ-Syn with SERT and DAT. This suggests that these proteins may interact directly or are at least in close enough proximity to allow for potential indirect protein-protein interactions at the cell membrane.

Despite the high degree of colocalization between β-Syn or γ-Syn and SERT/DAT, the cell surface expression of MATs remained unchanged. This supports the idea that β-Syn and γ-Syn do not seem to have major effects on MAT secretory trafficking or endocytosis-mediated internalization from the cell surface. Several studies have indicated synucleins may exert distinct regulatory effects on MATs via different trafficking mechanisms (reviewed by Oaks and Sidhu, 2011). While β-Syn or γ-Syn may influence early DAT trafficking stages, such as between the ER and the Golgi (Oaks et al., 2013), my results suggest that their overexpression does not significantly impact the integration of DAT or SERT into the plasma membrane or their overall distribution. Although the precise mechanisms of MAT trafficking were not the focus of this study, future investigations could employ more specific techniques to better assess surface expression. This could be performed through e.g. surface biotinylation using membrane-impermeable biotinylation reagents to provide more accurate measures of surface MAT levels by ensuring that only membrane-bound transporters are analyzed.

3.1.3 SNCA Knockout Mediates Reduction of SERT Activity in HEK293 Cells

The influence of *SNCA* knockout on serotonin reuptake was evaluated by disrupting *SNCA* expression in both HEK293 and N2a cells. Initial attempts to generate a knockout in N2a cells using a transiently expressed Cre-dependent CRISPR/Cas9 plasmid did not yield clear results. Therefore, an additional CRISPR-Cas9 gene editing experiment was carried out using sgRNA targeting murine *SNCA* (as outlined in **Materials and Methods**). However, these results were also inconclusive. Even though CRISPR-Cas9 is typically an effective method for gene editing, significant reductions in α-Syn protein levels could not be confirmed by Western blot, and *SNCA* mRNA expression levels were undetectable by qPCR. This is likely due to the characteristically low levels of endogenous α-Syn in N2a cells (Sofic, 2024). As a result, further propagation of N2a knockout clones was discontinued.

In contrast, HEK293 cells express higher levels of endogenous *SNCA* and can exhibit neuronal characteristics, making them a suitable model (Sofic, 2024, Shaw et al., 2002; Lee and Kamitani, 2011) Therefore, the CRISPR/Cas9-mediated knockout experiment was repeated in HEK293 cells. Western blot analysis confirmed the absence of α-Syn in knockout clone # 24 (KO24) and was propagated into a stable cell line. KO24 will be referred to as *SNCA* -/- HEK293 cells throughout the remainder of this discussion.

As hypothesized, the absence of α-Syn correlated with significant reduction in serotonin uptake (**Fig. 11A).** While this result aligns with expectations, I cannot exclude potential off-target effects resulting from the CRISPR-Cas9 knockout, as other chromosomal regions may have been disrupted. These off-target effects could (at least to a certain extent) be responsible for the observed effects in the uptake assessments. Genomic sequencing will be required to verify the specific INDELS generated by CRISPR-Cas9. Since V_{max} is dependent on surface expression of the transporter, these values should be interpreted with caution, especially considering potential changes in SERT surface expression. In *SNCA* -/- HEK293 cells, V_{max} was significantly reduced compared to WT control, even though equal amounts of SERT plasmid DNA were transfected. This shift in observed reduction of uptake activity is likely a direct consequence of the CRISPR-Cas9 mediated α-Syn knockout.

One key question that has not been fully addressed yet, is whether the changes observed in specific uptake assays (in response to α-Syn overexpression or knockout), are directly related to MAT trafficking and surface integration. Unlike β-Syn, which is missing most of the NAC region, α-Syn interacts with the C-terminus of DAT through its NAC region, which is a key feature of α-Syn as well as γ-Syn (Jakes et al., 1994; Uversky et al., 2002). This interaction could also influence the surface distribution and availability of the SERT. Similar protein-protein interactions have also been shown to affect the magnitude of MAT-mediated reuptake and MAT surface distribution under conditions of overexpression (Oaks & Sidhu, 2011; Lee et al., 2001). Therefore, the α-Syn knockout may limit essential interactions with SERT, leading to altered distribution of transporters at the cell surface and reduced reuptake capacity.

Throughout experiments, careful monitoring of cell counts, cell proliferation and maximal uptake velocities was maintained to minimize variability and avoid unintended consequences stemming from the knockout. Cell morphology and growth of *SNCA* -/- HEK293 cells were somewhat inconsistent during cell maintenance compared to WT cognate clones. This raises the question of whether the observed effect is directly related to α-Syn knockout or resulting

from an off-target effect. α-Syn knockout, as well as triple synuclein knockout studies in mice have suggested major phenotypes that do not seem to affect survival (Runwal and Edwards, 2021; Greten-Harrison et al., 2010). In this study, CRISPR-Cas9 edits were targeted to exon 4. It is possible that an accidental disruption of an unknown downstream gene occurred. Additionally, there is the unlikely chance that it not only modified the intended *SNCA* region, but another part of the genome. This could be affecting cell cycle, energy metabolism or cell communication.

Additionally, α-Syn was shown to regulate cell proliferation in dopaminergic neurons, where it appears necessary for the proper expression of essential genes for early neurogenesis (Prahl et al., 2022). As mentioned previously, *SNCA* -/- HEK293 cells occasionally struggled to divide during cell culture and maintenance. This suggests α-Syn may influence cell cycle regulation and cellular growth to some extent. In future studies, proliferation experiments could help clarify α-Syn's role in these processes. Regardless, the *SNCA* -/- HEK293 cells seemed to maintain their capability of differentiating into viable cells throughout multiple rounds of passage.

Despite close monitoring of cell counts, some variability in cell numbers was observed, which may have influenced experimental consistency. Protocols should be followed carefully to ensure control for cell viability and proliferation effects, especially during trypsinization. A study by Wersinger et al. (2004) reported that mild trypsinization reversed the α-Syn's negative regulation of DAT trafficking, leading to increased DA uptake by promoting more DAT at the cell surface. This suggests that proper cell adhesion is necessary for α-Syn to exert its regulatory effects on DAT. Careful attention should be given to the intensity and duration of trypsin treatments, as excessive trypsinization may disrupt synuclein-MAT interactions by affecting cell adhesion. This can be monitored through careful washing and centrifugation steps.

3.1.4 α-Syn Knockdown Reduces SERT Activity in HEK293 Cells

In addition to generating a *SNCA* -/- HEK293 stable cell line, RNAi-mediated gene silencing was employed to reduce the endogenous expression of α-Syn. Initially, this approach was attempted in N2a cells using siRNAs targeted against murine *SNCA*. However, several challenges were encountered, including contamination and low endogenous expression levels of murine *SNCA*. Species specificity of the siRNAs for murine *SNCA* yielded inconclusive results (data not shown). Due to these difficulties, the RNAi approach was shifted to HEK293 cells, which express higher levels of *SNCA*, as noted previously.

To validate the knockdown, three different siRNAs targeting human *SNCA* were evaluated in HEK293. Each siRNA demonstrated variability in its ability to reduce endogenous *SNCA* expression, as measured by corresponding reductions in serotonin reuptake using uptake assays and confirmed via Western blot analysis **(Fig 8).** Preliminary experiments revealed no significant differences between the negative control and the siRNA treated groups when analyzed by one-way ANOVA. Further experiments with additional replicates will be necessary to confirm the knockdown efficiency and reductions in reuptake reduction for the other siRNAs, as more statistical power is needed to fully validate these findings.

The RNAi-mediated knockdown experiments served as an alternative and complimentary approach to the CRISPR-Cas9 knockout experiments, with the goal of confirming or disproving the observed effects of α-Syn on SERT activity. Both approaches demonstrated that α-Syn negatively affects SERT activity by reducing serotonin uptake. However, whether this effect is contingent on changes in SERT surface expression remains to be determined. It is likely that the reduction in serotonin uptake observed after *SNCA* silencing correlates with a decrease in SERT expression at the cell surface. A previous study by Fountaine and Martins. (2008) showed that *SNCA* knockdown decreases DAT surface expression, leading to reductions in DA uptake. Their findings are in alignment with the outcomes of my siRNA experiments, which also demonstrated reduced monoamine reuptake. This further suggests that α-Syn plays a role in influencing MAT activity.

Interestingly, my findings are in accordance with the effects observed under α-Syn overexpression, where α-Syn increased SERT surface expression and amplified serotonin reuptake (Sofic, 2024). These results suggest dual roles are at play in regulating SERT activity; where knockdown reduces the transporters' ability to mediate reuptake and overexpression enhances it. Together these experiments broaden our understanding of how α-Syn modulates SERT activity, highlighting its role in regulating SERT surface expression and reuptake capacity.

3.1.5 Future Perspectives

Given the successful α-Syn gene silencing in HEK293 cells, future investigations could extend to siRNA knockdown experiments targeting β-Syn and γ-Syn in the same cell line. Combining the data from β-Syn and γ-Syn overexpression with findings from α-Syn (Sofic, 2024) could provide a comprehensive understanding of the collective role of the synucleins in modulating MATs. Western blot analysis showed minimal levels of α-Syn expression in N2a cells

compared to HEK293293 cells, where endogenous α-Syn levels were higher (Sofic 2024). However, the endogenous expression levels of β-Syn and γ-Syn in these cell types have yet to be determined. Establishing baseline expression levels of these synucleins would be an important starting point for evaluating their co-expression ratios and combined influence on synaptic function.

Given that β-Syn has been shown to colocalize with α-Syn in various neuronal types of the substantia nigra pars compacta (Li et al., 2002), it would be interesting to explore how the co-expression of all three synucleins affects monoamine uptake when overexpressed in vitro. For example, co-transfections of β-Syn or γ-Syn with α-Syn, or β-Syn and γ-Syn together could have variable effects on MATs. The triple knockout of all three synucleins in mice produced major phenotypes, including effects on terminal size and synaptic structure, and suggests that synucleins have overlapping and compensatory roles (Greten-Harrison et al., 2010). Future overexpression studies can be justified to understand whether co-expressing different combinations of synucleins results in unique regulatory effects on MAT activity when more than one synuclein is present. Moreover, research by Carnazza et al. (2022) showed that β-Syn and γ-Syn modulate the vesicle binding of α-Syn and reducing its activity at the synapse. This indicates some level of interplay between the synucleins. However, their physiological roles at or near the cell membrane are not completely defined in the presence or absence of one another. Fine titration experiments monitoring all three synucleins could clarify their individual contributions or compensatory roles in MAT regulation.

The *SNCA* knockout cell line generated during this thesis work sets the stage for further investigation into the physiological or pathological roles of α-Syn's in vitro. To verify whether CRISPR-Cas9 gene edits had the intended effect on *SNCA*, complementation studies should be explored. This would involve transfecting a functional plasmid copy of α-Syn back into the knockout cell line, which should rescue the genetic manipulations made to *SNCA*. Restoring the WT phenotype in the *SNCA* knockout cell line would be considered evidence of a successful CRISPR gene edit. Additionally, the cognate clone # 49 (WT from single cell cloning) may have generated some off target effects and rescue experiments should also be compared with actual WT HEK293 cells from ATCC. This would account for any off-target effects in the KO24 clone. Whether the knockout of α-Syn leads to permanent changes in serotonin uptake or if reintroducing α-Syn reverses these changes to WT levels, is yet to be determined.

While β-Syn and γ-Syn overexpression did not significantly affect monoamine uptake in this study, the effects of their overexpression in the opposite direction have yet to be examined. Employing a super-fusion system to study the influence of synuclein overexpression on reverse transport of SERT or DAT (*i.e.* induced by amphetamines), would help characterize neurotransmission in terms of both monoamine uptake and release. It would be informative to investigate whether fluctuations in monoamine release follow the same pattern of uptake. Since there can be a change in uptake but not release (or vice versa), the two events do not necessarily go "hand-in-hand" (Steinkellner et al., 2012), and should be independently characterized. Investigating this balance between monoamine release and uptake across various cell types could provide insights into synaptic regulation and their roles in synucleinopathies.

The extent to which synuclein toxicity contributes to neurodegeneration is still questionable. While aggregation of α-Syn appears to be toxic to neurons and is linked to neurodegeneration (Auluck et al., 2010), current literature has yet to confirm whether synuclein overexpression alone is entirely responsible for killing cells (Pinto-Costa et al., 2023). In a recent study by Moreno et al. (2024), α-Syn was overexpressed by injecting a novel adeno-associated viral vector (AAV) directly into the substantia nigra pars compacta, without inducing any observable dopaminergic neurodegeneration or loss of dopamine neurons. It remains unclear whether excessive α-Syn overexpression is toxic due to gain-of-toxic-function or arises from a loss-of-physiological-function. Presumably, α-Syn is perpetually being captured by amyloid plaques or consolidated into Lewy-bodies, which could prevent it from fulfilling an endogenous function. So, why would synuclein only exist to cause toxicity? The argument tends to be, that a loss-of-function, rather than gain of function is mediating toxicity. Yet, there must be an endogenous function, which remains largely undefined. These proposals and recent findings by Moreno et al. (2024) question the consensus that excessive α-Syn is toxic to the cell, and address issues pertaining to whether α-Syn overexpression alone is sufficient to induce neurodegeneration. Neurodegeneration may involve additional factors, such as being triggered by microglial activation (Hanslik et al., 2021).

Targeting α-Syn using antibodies is an important area of research. Studies by Näsström et al. (2011) demonstrated that monoclonal antibodies effectively reduce α-Syn levels. However, clinical studies aimed at lowering α-Syn levels (cinpanemab and prasinezumab) have so far shown no major improvement in clinical outcomes (Lang et al., 2022; Pagano et al., 2022). More recent work by Pagano et al. (2024) suggests that reducing α-Syn with prasinezumab may have potential benefits, but further research is necessary to determine therapeutic efficacy.

3.2 Summary and Conclusion

This thesis explored the roles of α-Syn, β-Syn, and γ-Syn in regulating MATs (particularly, SERT and DAT) at the cell surface. The observed increases in SERT surface expression due to α-Syn overexpression, may be attributed to of α-Syn's role in enhancing secretory trafficking, reducing endocytic trafficking, or directly regulating gene expression. These modulatory mechanisms might be less relevant for β-Syn or γ-Syn, potentially explaining their minimal effects on MAT surface expression. Despite high colocalization with MATs, β-Syn or γ-Syn did not significantly affect MAT surface expression, nor did they alter the reuptake of their respective monoamines.

The influence of α-Syn's influence on SERT-mediated reuptake was further investigated through gene silencing experiments targeting α-Syn. In contrast to overexpression, the reduction or loss of *SNCA* revealed a significant decrease in serotonin reuptake in HEK293 cells. These findings highlight α-Syn's duality in regulating SERT activity. Given the success of both siRNA knockdown and CRISPR-Cas9-mediated *SNCA* knockout in HEK293 cells, future studies on SERT surface expression are warranted for comparative evaluation. Thus far, the results reveal a clear contrast between β-Syn and γ-Syn's minimal effects and α-Syn's significant influence on MATs, suggesting distinct regulatory roles for each synuclein in MAT surface expression and monoamine reuptake. While α-Syn plays a direct role in modulating serotonin reuptake, β-Syn and γ-Syn have more limited roles and might be involved in other cellular processes, like chaperoning or trafficking mechanisms.

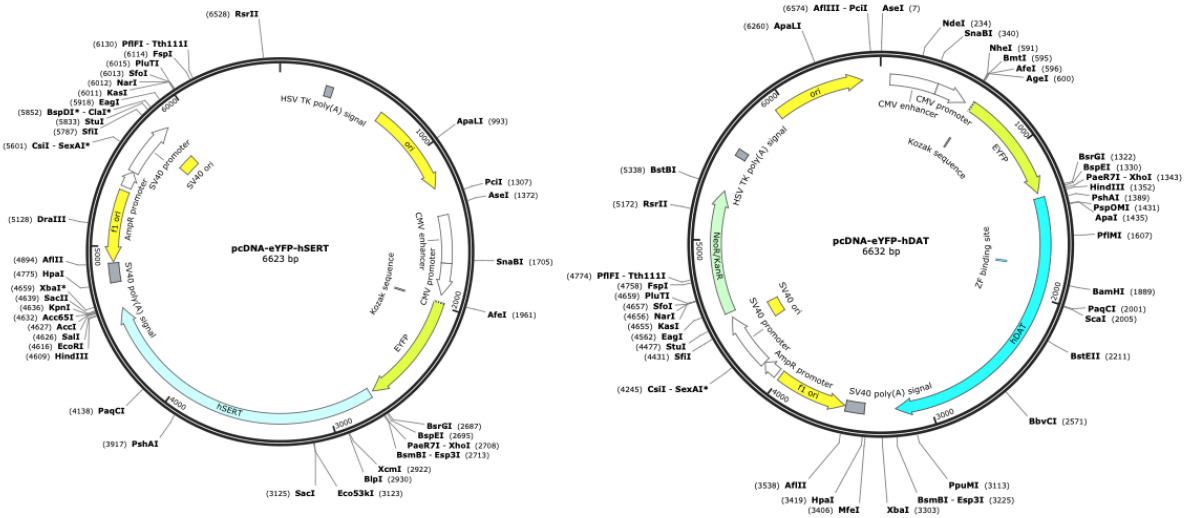
In conclusion, the findings of this study provide insight into the differential regulation of monoaminergic neurotransmission by synucleins. α-Syn uniquely modulates MAT activity by affecting cell surface expression and reuptake, while β-Syn and γ-Syn do not exert similar effects in N2a cells. However, neither the HEK293 nor N2a cell lines fully differentiated into synapse forming neurons under the conditions established throughout this study. Future research should focus on clarifying the interaction of synucleins with MATs in vivo. This would be necessary to confirm the collective findings from this study in a synaptic context. Ultimately, this thesis offers better understanding of the physiological roles of the synucleins at the synapse and highlight their complexity in the modulation of monoamine homeostasis, particularly through MAT-mediated reuptake.

4 Materials and Methods

4.1.1 Cell Culture, Maintenance, and Transfections

HEK293 and Neuro2a (N2a) cells (originally obtained from ATCC) were cultured in high glucose Dulbecco's modified Eagle's glucose growth medium ((DMEM) - Capricorn Scientific, Germany). The growth media was supplemented with 10% fetal bovine serum (FBS) and 100 µg/ml of streptomycin/penicillin. Cells were maintained in 5% CO_2-humidified air at 37°C and were passaged weekly. Before passaging, cells were washed with phosphate buffered saline (PBS: NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 4.3 mM, $KH2PO_4$ 1.5 mM), and detached using 10% trypsin(v/v). Cells were resuspended in DMEM to achieve the desired confluency for subsequent transient transfections. To ensure a high level of transgene expression, cells were passaged fewer than 40 times prior to transfections. Low-passage cell stocks were frozen at -80°C and thawed as needed. Cultures were routinely screened for contamination by microscopy before proceeding with further experiments.

For transfections, HEK293 and N2a cells were seeded into 6-well (35 mm) or 10 cm dishes and grown overnight to 70% confluency. Plasmids for transfections were previously generated by members of the Steinkellner lab using molecular cloning techniques. Briefly, each respective cDNA (eYFP-hSERT or eYFP-hDAT, β/γ-Syn-V5, or wild-type human alpha-synuclein = hASyn) was amplified by PCR and inserted into a pAAV-hSyn1-DIO (double inverted open reading frame at AscI restriction sites.



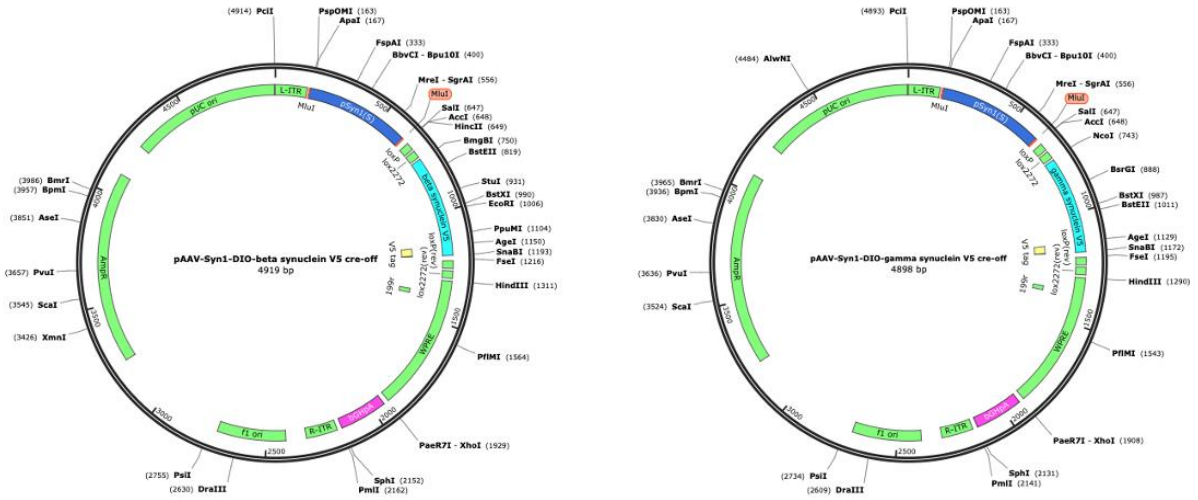


Figure 14: DNA plasmid maps of constructs used. *Top:* SERT/DAT. *Bottom:* β/γ-Synuclein.

For co-transfections, increasing concentrations of β-Syn, and γ-Syn (both V5-tagged) plasmid DNA were used along with a constant concentration of eYFP-hSERT/DAT plasmid DNA (0.5 µg for 6-well plates or 2.5 µg for 10 cm dishes). Cells were transiently transfected using jetPRIME transfection reagent (Polyplus Transfection Inc., USA), with total DNA adjusted using 'empty vector' (pcDNA3.1) up to a final concentration of 2 µg per 35 mm well (6 well plate) or 10 µg per 10 cm dish. The desired expression ratios of hSERT:Synuclein were 1:0, 1:1, 1:2 and 1:3, respectively. Transfections were performed by adding the jetPRIME-DNA mixture (briefly vortexed and incubated at room temperature for 10 minutes) to the cells dropwise. After four hours, the transfection medium was replaced with fresh DMEM, and cells incubated for an additional twenty hours before further analysis.

4.1.2 Seeding and Plate Preparations

Twenty-four hours after transfection, YFP-SERT or YFP-DAT expression was confirmed using fluorescence microscopy to assess transfection efficiency. Cells were then trypsinized, centrifuged at 800 x g for 5 min at room temperature, and resuspended in fresh media. For single-concentration specific uptake assays, approximately 6 x10^5 cells per well were seeded in triplicate onto a 0.1% poly-D-lysine (PDL) precoated 48-well plate. Two additional wells per condition were reserved for cell counting. Simultaneously, cells from the same transfected suspension were seeded for other experiments; 6-well plates or 10cm dishes were used for preparing cell lysates for Western blot analysis, 24-well plates were used for immunocytochemistry experiments. Seeding densities were adjusted to 70% confluency for

optimizing fluorescence imaging for immunocytochemistry, while wells for cell lysate preparations were seeded to full confluency. All three experiments (uptake assays, immunocytochemistry, and Western blotting) were performed in parallel and completed on the same day (forty-eight hours post transfection) to allow adequate time for transgene expression.

4.1.3 [^{3}H]5-HT and [^{3}H]DA Uptake Assays

Forty-eight hours after transfection, cells were assayed for [^{3}H]5-HT and [^{3}H]DA uptake. Briefly, cells were washed and kept in 200 µL of room temperature Krebs-HEPES buffer (KHB); containing 10 mM HEPES, 120 mM NaCl, 3.0 mM KCl, 2.0 mM $CaCl_2$, 2.0 mM $MgCl_2$, with 2.0 mM D-(+)-glucose monohydrate (pH adjusted to 7.3). Prior to the uptake assay, a preincubation solution containing 10 µM paroxetine (SERT-inhibitor) in 100 µL of KHB was added to wells designated for nonspecific uptake for 5 min at 37°C.

For the uptake assay, final assay concentrations were prepared by diluting 10 µM paroxetine and 0.1 µM [^{3}H]5-HT or [^{3}H]DA (specific activity: 28-50 Ci/mmol) in KHB. Radiolabeled monoamines supplied by Perkin Elmer Life Sciences, (Germany). Each well received 100 µL of assay mixture, and uptake was measured for one minute for 5-HT, and two minutes for DA. Uptake was terminated by aspirating assay mixture, followed by rapidly washing the cells three times with ice-cold KHB. Non-specific uptake was determined in the presence of 10µM paroxetine after five-minute preincubation. For saturation uptake kinetic assays (to determine the K_m and V_{max} for serotonin transport via SERT), the specific activity of 0.1µM [^{3}H]5-HT was adjusted by adding increasing concentrations of unlabeled 5-HT (0.1- 30.0 µM) in KHB. Non-specific uptake was determined in the presence of 10µM paroxetine after a 5 min preincubation at 37°C. All subsequent assay procedures followed the same protocol as described above.

After uptake was completed, cells were solubilized in 0.5 mL of 1% SDS at room temperature and transferred to vials containing 2mL scintillation cocktail (Rotiszint eco plus, Germany). A liquid scintillation analyzer (Perkin Elmer Life Sciences, Germany) was used to measure the amount of [^{3}H] isotope incorporated into the cells, expressed as counts per minute(cpm). Two or three wells per condition were reserved for cell counting using a Neubauer chamber. Prior to counting, wells were washed twice with KHB buffer, trypsinized, and incubated at room temperature.

4.1.4 Immunocytochemistry

Cells were seeded onto 24-well plates and fixed with 4.0% paraformaldehyde (PFA) for 15 minutes at room temperature. PFA was rinsed off with PBS, and cells were then blocked using 5.0% Normal Donkey Serum (NDS) permeabilized with 0.1% Triton X-100 in PBS (blocking buffer) for one hour at room temperature. After blocking, the buffer was removed, and cells incubated with primary antibodies diluted in blocking buffer overnight at 4°C on a gentle shaker.

Primary antibodies included:

- Monoclonal mouse anti-SERT
  (1:500, self-made; *see* Montgomery, Steinkellner et al., PMID: 24790205).
- Monoclonal rat anti-DAT antibody
  (1:500, MAB369, Millipore).
- Monoclonal rabbit anti-V5-tag D3H8Q antibody
  (1:1000, #13202, Cell Signaling Technologies, USA).

The following day, cells were washed, three times with PBS for ten minutes each, and then incubated with secondary antibodies diluted in blocking buffer for 2 hours at room temperature. Secondary antibodies provided by Jackson Immuno included:

- Donkey anti-mouse IgG conjugated to Alexa Fluor 594 (1:1000)
- Donkey anti-rat IgG conjugated to Alexa Fluor 594 (1:1000)
- Donkey anti-rabbit IgG conjugated to Alexa Fluor 488 (1:1000)

After incubation with secondary antibodies, cells were washed three times with PBS, counterstained with DAPI for 1 min, and left in a small volume of PBS for imaging. Fluorescence images were captured using a confocal microscope equipped with a digital camera. Brightness, contrast, and exposure times were held constant for each image within an experiment. Two to three fields of view were photo-documented using 10x, 20x and 40x objective lenses.

4.1.5 SNCA Knockout with CRISPR-Cas9 Gene Editing

CRISPR/Cas9 gene editing was performed using a predesigned single guide RNA (sgRNA) from Integrated DNA Technologies, targeting exon 4 of human *SNCA*, to generate *SNCA -/-* and cognate WT cells from a HEK293 cell line. The Design parameters were as follows:

Prior to transfection, a duplex of Cas9 and cr/tracrRNA (Hs.Cas9.SNCA1.AA) CRISPR-Cas9 crRNA/ALT-R CRISPR-Cas9 tracrRNA) was annealed and resuspended in RNase-free water to a final concentration of 1 μM. HEK293 cells were cultured in DMEM supplemented with 10% FBS (without antibiotics) and seeded in 24-well plates to reach approximately 80% confluence on the following day. The original growth factored-supplemented culture medium was reserved for later use in expanding the cells. Once confluency was reached, cells were transiently transfected using Lipofectamine CRISPRMAX (Invitrogen) with positive strand sgRNA 5' - GTCCTTTTTGACAAAGCCAG - 3' targeting exon 4 (anti-sense strand) of human *SNCA* in HEK293 cells. Brief transfection procedure: A solution of 21 μl of OptiMEM, 27 μl of Cas9 Nuclease (1μM), and 27 μl of cr/tracrRNA (1 μM) was mixed and vortexed. Separately, 7.5 μl of Cas9 PLUS reagent (Invitrogen) was added to the Cas9 protein/gRNA solution and incubated for 5 min at 25°C. In a second tube, 75 μl of OptiMEM was mixed with 6 μl of Lipofectamine CRISPRMAX (Invitrogen). The Cas9/gRNA solution was then transferred to the Lipofectamine CRISPRMAX mixture and incubated for 10 min at 25°C. The final combined solution (50 μl) was added dropwise to each well of the 24-well plate.

The day after transfection, cells were transferred to 6 cm expansion dishes for expansion and monitored for three days to ensure proper growth and absence of contamination. Cultures were then resuspended in the previously reserved growth-factored supplemented medium, and single-cell clones were manually isolated and transferred into 96-well plates. After two weeks, single cell clones were identified using a microscope (4x/0.10 objective lens), and approximately fifty clones with suitable morphology and growth behavior were selected for further analysis. These single cell clones were transferred to 24-well plates, along with original growth medium, where their morphology and growth were monitored over the following days. Of the original fifty clones, thirty-three wells were selected for expansion into 6-well plates. Cell lysates from selected wells were prepared and analyzed for *SNCA* protein expression via Western blotting. From this pool of candidates, four different single-cell clones (including two cognate WT clones) were propagated for further evaluation. Western blot analysis confirmed

efficient knockout of *SNCA* in single cell clones (*see* **Figure 10A**). One single cell clone was subsequently propagated into a stable *SNCA* -/- cell line. A single WT cognate control clone, showing minimal reduction in α-Syn protein expression, was also selected for further experiments. Both *SNCA* -/- clone and WT cognate control were used in subsequent specific uptake assessments.

4.1.6 Assessment and Screening of siRNAs for Knockdown Efficacy

Knockdown of endogenous α-Syn in HEK293 and N2a cells was performed using predesigned siRNA oligonucleotides (Invitrogen, USA), targeted against human or murine-specific *SNCA*. A stealth RNAi (medium GC) duplex, unrelated to the vertebrate transcripts, was used as a negative control to minimize sequence homology. The sequences of three different siRNA oligonucleotides are outlined below:

SNCAHSS144005 (CAT# 10620318/19; 503860 **A10/B01**)

ss (sense strand) 5' - UCAAGACUACGAACCUGAAGCCUAA - 3' **(A10**)

as (antisense strand) 5' - UUAGGCUUCAGGUUCGUAGUCUUGA - 3' (**B01**)

SNCAHSS144006 (CAT# 10620318/19; 506239 **B12/B05)**

ss 5' - GGGAGUUGUGGCUGCUGCUGAGAAA - 3' (**B12**)

as 5' - UUUCUCAGCAGCAGCCACAACUCCC - 3' (**B05**)

SNCAHSS144008 (CAT# 10620318/19: 506239 **A06/B10**)

ss 5' - GACCAAAGAGCAAGUGACAAAUGUU - 3' **(A06**)

as 5' – AACAUUUGUCACUUGCUCUUUGGUC - 3' **(B10**)

The functional effects from *SNCA* knockdown were assessed using a modified serotonin uptake assay; conducted across two independent transfection days. Lyophilized siRNA duplexes were resuspended in RNase-free water to a final concentration of 20 μM and stored as stock solutions at -20°C. Prior to transfection, cells were seeded in 48-well plates (precoated with 0.2% PDL) in antibiotic free medium and grown to 40% confluency overnight (approximately 15,000-20,000 cells/well). The following day, cells were transfected with stealth siRNA duplexes and negative controls using Lipofectamine RNAiMAX reagent (Invitrogen, USA) at a final concentration of 10 nM. Briefly, siRNAs and RNAiMAX were resuspended in OptiMEM (Life Technologies Corporation, USA) incubated for 5 min at room temperature, and then the

siRNA:LFRNAiMAX complex was added to each well according to the manufacturer's instructions.

The next day, the growth medium was replaced with fresh DMEM containing antibiotics, and cells were monitored for viability using a light microscope. Forty-eight hours after the initial siRNA transfections, a secondary transfection with eYFP-hSERT plasmid (0.5 µg of DNA per well), was performed using Lipofectamine 2000 (Invitrogen, USA), thereby standardizing the experiment. Briefly, SERT:LF2000 complexes were combined in OptiMEM, incubated for twenty minutes at room temp, and then added to the wells. In parallel, a set of uncoated 6-well plates was seeded and transfected using the same RNAiMax protocol to prepare cell lysates for further analysis. Adjustments were made as per manufacturer's recommendations. Cells were assayed for serotonin uptake twenty-four hours after the secondary transfection, and cell lysates were prepared to evaluate α-Syn protein expression levels via Western blotting.

4.1.7 Western Blotting

Transfected cells were lysed on ice using radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with protease inhibitors (Roche complete). The RIPA buffer was composed of 150 mM NaCl, 10 mM EDTA, 0.1% SDS, 01% Triton X-100, 0.5% sodium deoxycholate, and 50 mM Tris-HCL (pH7.4). Lysates were rotated at 4°C for twenty min, and the soluble protein was separated from insoluble detergent fractions by centrifugation at 14,000rpm for 10 min at 4°C. Supernatants were collected and stored at -20°C until further use. Protein concentrations were quantified using the Pierce BCA protein assay (Thermo Fischer Scientific). Cell lysates (20-40 µg of protein) were resolved in 6X Laemmli buffer (375 mM Tris-HCL, 12% SDS, 40% glycerol, 0.6 M dithiothreitol, 0.06% bromophenol blue, and 5.0% beta-mercaptoethanol, (pH 6.8). Depending on the experiment, proteins were denatured at 50°C for 15 min (for evaluation of SERT and DAT) or at 95°C for 5 min for the synucleins. The samples were resolved on either 10-12% sodium dodecyl sulfate polyacrylamide gels via electrophoresis (SDS-PAGE) and electro-transferred onto nitrocellulose membrane using the semi-dry Trans-Blot Turbo Transfer System (Bio-Rad). Note, wet transfer was specifically used for α-Syn blotting. For synucleins, membranes were fixed with 0.4% PFA for 30 minutes at room temperatures, and Ponceau S staining was performed to confirm equal loading across lanes.

Membranes were blocked for one hour at room temperature in 5% Nonfat dry milk (NFDM) in Tris buffered saline containing 0.1% Tween-20 (TBST). After blocking, membranes were

washed three times with TBST and incubated overnight at 4°C with primary antibodies diluted in TBST:

- Monoclonal rabbit anti-V5-tag D3H8Q antibody (1:2000, #13202, Cell Signaling Technologies, USA).
- Polyclonal rabbit anti-GFP D5.1 antibody (1:5000, A11122, Invitrogen, USA)
- Monoclonal mouse anti-α-Syn 4D6 antibody (1:1000, Biolegend, USA)
- Monoclonal rabbit anti-GAPDH D16H11 antibody (1:2000, 50174S, Cell Signaling Technologies, USA).

The following day, membranes were washed three times with TBST and incubated with the following secondary antibodies, diluted in blocking solution for 2 hours at room temperature:

- Goat anti-mouse IRDye 680RD (1:5000, LI-COR, USA).
- Donkey anti-rabbit IRDye 800CW (1:5000, LI-COR, USA).

After three additional washes with TBST and once with TBS, fluorescence was detected using an Odyssey infrared imaging system (LI-COR). Membranes were subsequently probed with a primary antibody rabbit anti-GAPDH (1:2000, Cell Signaling Technologies) as a loading control, followed by detection with Donkey anti-rabbit IRDye 800CW (1:5000, LI-COR, USA). Fluorescence intensities were quantified using ImageJ software (version 2009), normalized to GAPDH, and expressed as intensity per unit area.

4.1.8 RNA Isolation and qPCR

RNA was isolated using the NucleoSpin RNA kit (MACHERY-NAGEL, Germany) according to the manufacturer's instructions. Total RNA concentrations were measured using a NanoDrop 200 spectrophotometer (Thermo Fisher). For cDNA synthesis, 1.0 µg of total RNA per sample was was reverse transcribed using the Revert Aid First Strand kit (Thermo Fissher), with oligo(dT) primers. The reverse transcription reaction was incubated on a thermal cycler at 42°C for 1 hour and terminated at 70°C for 5 minutes. The cDNA was stored at -20°C until further use. qPCR reactions were prepared according to manufacturer's protocol using SYBR Green Master Mix (BIO-RAD). Briefly, serial dilutions of cDNA samples were prepared, and specific forward and reverse primers were added to each reaction. The reactions were run on a BIO-RAD thermal cycler. The following primers were used:

GAPDH

- TS64(fw) Forward - CATCACTGCCACCCAGAAGACTG
- TS65(rv) Reverse - ATGCCAGTGAGCTTCCCGTTCAG

SNCA

- TS58fw - CAGAGGCAGCTGGAAAGACA
- TS59rv – CACCACTGCTCCTCCAACAT

4.1.9 Statistical Analysis

Results were analyzed using Microsoft Exel and GraphPad Prism software version 10 (GraphPad Software Inc., USA). Histograms and relevant statistical analyses were also generated using GraphPad Prism. The number of experiments (n) refers to the number of independent experiments conducted ('biological replicates'). Counts per minute data were used to calculate absolute values, expressed as pmol/millioncells/minute. Nonspecific uptake measurements (usually corresponding to less than 10% of total uptake) were subtracted from the total uptake to determine specific uptake values. Triplicate measurements were averaged for each experiment and are presented as mean of the means ± SEM (all data presented as absolute values unless normalized to control). Statistical comparisons for single-concentration uptake assays and knockdown/knockout experiments were performed using one-way analysis of variance (ANOVA), followed by post hoc multiple comparisons tests as indicated. P-values of less than 0.05 were considered statistically significantly, and significant differences are noted in figures. Kinetic parameters, including V_{max} and K_m were derived from a Michaelis-Menten least squares regression curve fit. An extra-sum-of-squares F test was conducted to compare parameters between *SNCA* -/- and cognate WT control samples.

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