

**Understanding the consequences of chronic nuclear alpha-synuclein mislocalization in mice
and mapping alpha-synuclein cell-specific topography**

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Dedication

This thesis is dedicated to the mice whose lives were given in service to science and to all those who donated their brain in the pursuit of scientific knowledge. Their selflessness and tireless commitment continue to serve as a guiding light, inspiring us to strive for breakthroughs that will alleviate suffering and improve the lives of countless individuals. In honouring their memory, may we uphold the principles of inquiry, compassion, and collaboration, ensuring that their legacy endures in the progress we make towards conquering the challenges of illness and disease.

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Abstract

Parkinson's disease (PD) is the fastest growing neurodegenerative disease worldwide and is characterized by dopaminergic neurodegeneration and the accumulation of intraneuronal alpha-synuclein (α Syn) aggregates. While α Syn is typically known as a presynaptic protein, a small population resides in the nucleus. In PD, nuclear α Syn has been shown to increase relative to age-matched controls, and animal models of nuclear α Syn overexpression exhibit neurotoxicity. However, there is an endogenous population of nuclear α Syn that has been shown to be beneficial in repairing DNA damage, therefore it remains unclear whether the nuclear accumulation of α Syn is neurotoxic or neuroprotective. Previous work modeling the nuclear accumulation of α Syn rely on its overexpression, however α Syn is inherently aggregate-prone, thus clouding phenotypes directly attributable to nuclear α Syn. In this dissertation, we model nuclear α Syn accumulation in a novel mouse model (*Snca*^{NLS}), which mislocalizes endogenous α Syn into the nucleus without relying on its overexpression. *Snca*^{NLS} mice exhibited significant age-dependent motor deficits, cortical neurodegeneration, and decreased survival rates compared to littermate controls. To gain further insight into the mechanism(s) of nuclear α Syn toxicity, we profiled the nuclear α Syn interactome using TurboID, a proximity labelling assay, in primary cortical neurons. Interestingly, we noted an enrichment of spliceosome and RNA binding proteins and validated Luc7l, an RNA-binding protein, to interact with α Syn and modulate its protein levels. Serendipitously, the *Snca*^{NLS} model was also found to be an excellent reporter allele for mapping α Syn topography throughout the mouse. Identifying α Syn topography has been challenging due to its largely pre-synaptic locale, which are notoriously difficult to image

and correlate to cell bodies. With α Syn in the nucleus, we were more easily able to map α Syn⁺ cell density and intensity throughout the brain, spinal cord, retina, and gut. We chronicled the topographical distribution of α Syn across different brain structures, cell types, and throughout the periphery. Surprisingly, all neurons in the stomach and duodenum showed some level of α Syn expression, however to different extents, highlighting the heterogeneity of neurons in the gut. This thesis explores the consequences of constitutive nuclear α Syn accumulation, begins to unravel the molecular mechanisms behind this toxicity, and documents the localization of α Syn-abundant cell types. Altogether, this thesis aims to improve our understanding of α Syn, which will enable a deeper insight into PD and other α -synucleinopathies, ultimately leading to more efficient therapeutics.

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List of Abbreviations

-/-	gene knockout
5-HIAA	5-hydroxyindoleacetic acid
6-ODHA	6 hydroxydopamine
AAV	adeno-associated virus
AMB	nucleus ambiguus
APEX	ascorbate peroxidase
α Syn	alpha-synuclein protein
BiFC	bimolecular fluorescence complementation
CK2	casein kinase 2
CNS	central nervous system
COMT	catechol-O-methyltransferase
DA	dopamine
DAB	diaminobenzidine
DaT-SPECT	dopamine transporter single photon emission computed tomography
DLB	dementia with Lewy bodies
DMX	dorsal motor nucleus of the vagus nerve
DOPAC	3,4-dihydroxyphenylacetic acid
ENC	enteric neuron class
ENS	enteric nervous system
ER	endoplasmic reticulum
ESCRT-III	endosomal sorting complex required for transport III
GRK	G protein-coupled receptor kinase
GWAS	genome-wide association study
HVA	homovanillic acid
H&E	hemotoxylin and eosin
H&Y	Hoen and Yahr
IP	immunoprecipitation
LB	Lewy body

LC	locus coeruleus
LC-MS	liquid chromatography-mass spectrometry
LN	Lewy neurite
LRN	lateral reticular nucleus
MAO-B	monoamine oxidase B
MAP1B	microtubule-associated protein 1B
MDS-UPDRS	movement disorder society unified Parkinson's disease rating system
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRgFUS	magnetic resonance imaging-guided focused ultrasound
MRI	magnetic resonance imaging
MSA	multiple system atrophy
MSN	medial septal nucleus
NAC	non-amyloid compartment
nbM	nucleus basalis of Meynert
NEFL	neurofilament triplet L protein NES
NLS	nuclear localization signal
NSAID	non-steroid anti-inflammatory drugs
NSF	<i>N</i> -ethylmaleimide-sensitive fusion
OPC	oligodendrocyte precursor cells
PD	Parkinson's disease
PDD	Parkinson's disease with dementia
PFF	pre-formed fibril
PLK2	Polo-like kinase 2
PPN	pedunculopontine nucleus
PTM	post-translational modification
REM	rapid eye movement
SNARE	soluble <i>N</i> -ethylmaleimide-sensitive fusion attachment protein receptor
SNc	substantia nigra <i>pars compacta</i>
SNCA/Snca	alpha-synuclein gene
ssODN	single-stranded oligodeoxynucleotide
TH	tyrosine hydroxylase
TRIM28	tripartite motif-containing 28
VTA	ventral tegmental area

List of Manuscripts

1. **Geertsma HM**, Suk TR, Ricke KM, Horsthuis K, Parmasad JLA, Fisk ZA, Callaghan SM, Rousseaux MWC. Constitutive nuclear accumulation of endogenous alpha-synuclein in mice causes motor impairment and cortical dysfunction, independent of protein aggregation. *Hum Mol Genet.*, Volume 31, Issue 21, 1 November 2022, Pages 3613-3628, <https://doi.org/10.1093/hmg/ddac035>
2. **Geertsma HM**, Brain D, Nguyen B, Callaghan SM, Rousseaux MWC. Luc7l interacts with and stabilizes alpha-synuclein in the nucleus. *In preparation.*
3. **Geertsma HM***, Fisk ZA*, Sauline L*, Prigent A, Kurgat K, Callaghan SM, aSCENT-PD consortium, Henderson MX[#], Rousseaux MWC[#]. A topographical atlas of α Syn dosage and cell-type expression in the mouse brain and periphery. *npj Parkinson's Disease*, Volume 10, Issue 65, 19 March 2024, <https://doi.org/10.1038/s41531-024-00672-8>

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List of appended articles

1. **Geertsma HM**, Rousseaux MWC. Convergent systems-based approaches identify a role for OCIAD1 in Alzheimer's disease. *EBioMed*, Volume 52, 102627, February 2020, <https://doi.org/10.1016/j.ebiom.2020.102627>
2. **Geertsma HM**, Ricke KM, Rousseaux MWC. Assessment of dopaminergic neurodegeneration in mice. *Methods Mol Biol.*, Volume 2515, Pages 151-169, July 2020, https://doi.org/10.1007/978-1-0716-2409-8_10
3. Suk TR, Nguyen TT, Fisk ZA, Mitkovski M, **Geertsma HM**, Parmasad JLA, Heer MM, Callaghan SM, Benseler F, Brose N, Tirard M, Rousseaux MWC. Characterizing the differential distribution and targets of Sumo1 and Sumo2 in the mouse brain. *iScience*, Volume 26, Issue 4, 21 April 2023, 106350, <https://doi.org/10.1016/j.isci.2023.106350>
4. Parmasad JLA*, Ricke KM*, Nguyen B, Stykel MG, Buchner-Duby B, Bruce A, **Geertsma HM**, Lian E, Lengacher NA, Callaghan SM, Joselin A, Tomlinson JJ, Schlossmacher MG, Stanford WL, Ma J, Brundin P, Ryan SD, Rousseaux MWC. Genetic and pharmacological reduction of CDK14 mitigates synucleinopathy. *Cell Death & Disease*, Volume 15, Article number 246, 04 April 2024, <https://doi.org/10.1038/s41419-024-06534-8>

Chapter 1: Introduction

1.1 Overview of Parkinson's disease

Parkinson's disease (PD) is the most common movement disorder and is the fastest growing neurodegenerative disease worldwide (Bach et al., 2011; Dorsey et al., 2018). While there are many symptoms of PD, the most well-known are the cardinal motor symptoms: tremor, rigidity, akinesia/bradykinesia, and postural instability. Although PD was initially described in 1817, our understanding of the underlying mechanisms of disease remains unknown (Parkinson, 1817). The two histological hallmarks of disease are dopaminergic neurodegeneration and alpha-synuclein (α Syn) proteinaceous aggregates termed Lewy bodies (LBs) and Lewy neurites (LNs; Carlsson et al., 1957; Spillantini et al., 1997). However, it remains unclear precisely why dopaminergic neurons degenerate, α Syn aggregates, or the role of LBs in disease. Moreover, there are no disease-modifying therapeutics, only treatments targeting symptom management. While the later stages of disease have been well-documented through histological hallmarks and disease symptoms, little is known about the causes and early manifestations of PD. Gaining a better understanding of these earlier timepoints will aid in the development of disease-modifying treatments to improve the lives of the millions that have been diagnosed with PD worldwide.

1.2 Clinical manifestations of PD

People live with PD for decades and begin to show symptoms decades before diagnosis. The symptoms of PD are initially subtle, and patients can experience a broad range of disease

manifestations throughout their lifetime. However, there are some key hallmarks of PD symptomology that have been staged based on disease severity.

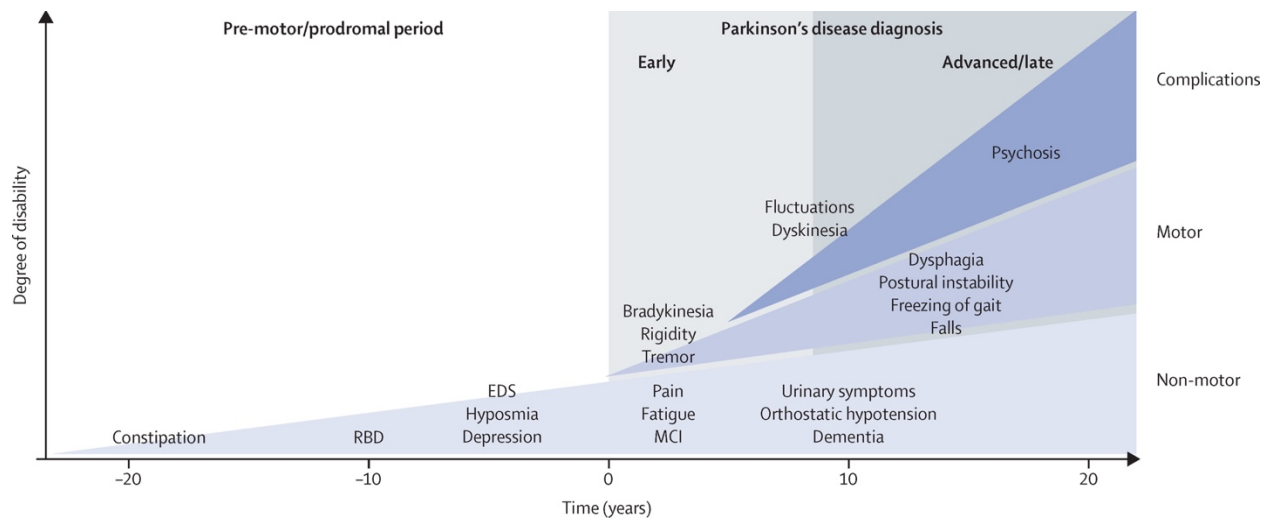


Figure 1.1: Clinical symptoms and time course of Parkinson's disease progression (from Kalia & Lang, 2015). Plotting symptom presentation across time relative to the diagnosis of Parkinson's disease. EDS = excessive daytime sleepiness, MCI = mild cognitive impairment, RBD = rapid eye movement (REM) sleep behaviour disorder.

1.2.1 Motor symptoms

The four hallmark symptoms of PD can be remembered using the mnemonic TRAP: tremor, rigidity, akinesia/bradykinesia, and postural instability. Initially, PD is diagnosed based on the emergence of at least two of these hallmarks. Other motor features of PD include gait and balance alterations, hypokinetic dysarthria (monotonous speech pattern/volume, increased speech rate), and altered reflexes (Kalia & Lang, 2015; Tolosa et al., 2021; Atalar et al., 2023). These symptoms typically worsen with time, in concert with dopaminergic neurodegeneration.

1.2.2 Non-motor symptoms

Some non-motor symptoms of PD tend to present years prior to diagnosis, while others appear later in disease progression. Before diagnosis, a majority of PD patients will experience constipation, hyposmia, and/or rapid eye movement (REM) sleep behaviour disorder (RBD; Kalia & Lang, 2015; Tolosa et al., 2021). Individuals with PD may also experience urinary dysfunction, depression, anxiety, excessive daytime sleepiness, oropharyngeal dysphagia (difficulty swallowing), and mild cognitive impairment (Kalia & Lang, 2015; Kwon & Lee, 2019; Tolosa et al., 2021). In late disease stages, patients may begin to experience a worsening of these non-motor symptoms, in addition to dementia and psychosis (Kalia & Lang, 2015; Tolosa et al., 2021). Recent research has been focused on identifying the early non-motor symptoms or cellular pathology as potential biomarkers for PD.

1.3 PD incidence and etiology

The incidence of PD is ~10-20 per 100,000 people per year and is typically diagnosed in one's 60s (Tysnes & Storstein, 2017). PD has a male bias of ~1.5x and the most significant risk

factor for idiopathic PD is age, with risk increasing 10-fold from 0.4% to 4% between 50-80 years of age (Berg et al., 2015).

The vast majority of PD patients, ~85%, have sporadic or idiopathic PD, meaning disease stems from *de novo* mutations, environmental exposures, or some unknown cause (Inamdar et al., 2007; Tysnes & Storstein, 2017; Willis et al., 2022). There is a large heterogeneity in PD patients, including in their symptom onset, presentation, and progression, in addition to their protein pathology and neurodegeneration. This disparity can be attributed to the possible interplay between genetics, epigenetics, and environmental exposures that may underly PD pathophysiology (Lill, 2016).

1.3.1 Idiopathic PD

Epidemiological studies have found pesticide exposure and rural living (including agricultural occupation and drinking well water), the use of beta blockers, heavy metal exposure, and infections from bacteria, fungi, or viruses, to all increase the risk of developing PD (Foubert-Samier et al., 2012; Goldman et al., 2012; Kalia & Lang, 2015; Noyce et al., 2012; Delic et al., 2020; Fletcher et al., 2012; Patrick et al., 2019; Pyatha et al., 2022; Wang et al., 2020). These studies have also identified environmental factors that correlate with a decreased risk in developing PD including smoking tobacco, drinking coffee, using non-steroid anti-inflammatory drugs (NSAIDs) and calcium channel blockers, and consuming alcohol (Kalia & Lang, 2015; Noyce et al., 2012; Li et al., 2022; Mappin-Kasirer et al., 2020; Ren & Chen, 2020). Researchers have tried to make sense of these environmental exposures and how they contribute to disease risk through prediction formulas that tabulate factors such as age, sex, genetics, common prodromal symptoms, and environmental exposures (Berg et al., 2015; Li et al., 2022; Schlossmacher et al., 2017). Here, they provide different weights to each aspect of PD

risk to model and quantify disease incidence. However, more work is needed to completely delineate PD risk and garner a better understanding of what could be causing what we currently classify as idiopathic PD.

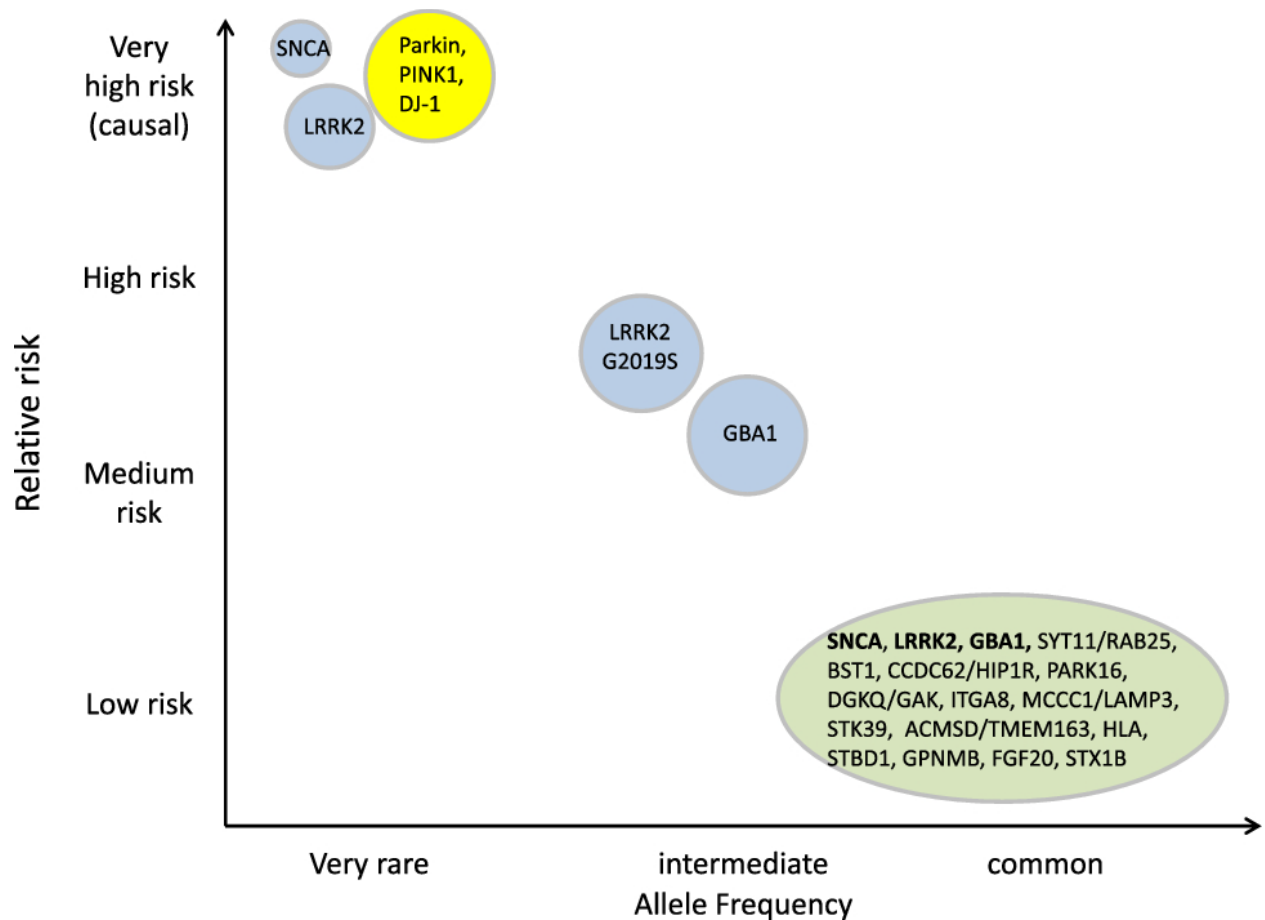


Figure 1.2: Genetic architecture of Parkinson's disease (from Gasser, 2023). Illustrating rare mutations in genes that confer a high risk of developing disease, versus more common genetic mutations that confer a lower risk of developing disease.

1.3.2 Familial PD

The first familial form of PD was identified from an Italian family and three Greek kindreds that were found to have a G209A mutation in the *SNCA* gene that led to an alanine to threonine substitution at position 53 (*SNCA*^{A53T}; Polymeropoulos et al., 1997). With the widespread increase of sequencing efforts, more and more genetic causes or risk factors for PD are being identified (Billingsley et al., 2018; Huang et al., 2024). Monogenic forms of PD can be broken down into two patterns of inheritance: autosomal dominant and autosomal recessive.

1.3.3 Autosomal dominant PD genes

There are several genes that, when mutated or multiplied, elicit monogenic, autosomal dominant PD, including *SNCA*, *LRRK2*, *VPS35*, and *RAB32*. There are several different documented families with *SNCA* mutations or multiplications. *SNCA* triplications have been documented in a small handful of patients and families, and have been reported to cause early-onset, aggressive parkinsonism with cognitive and autonomic dysfunction (Ferese et al., 2015; Olgiati et al., 2015; Singleton et al., 2003). While *SNCA* triplications are fully penetrant, *SNCA* duplications are partially penetrant and do not always lead to the same early-onset PD symptomology (Chartier-Harlin et al., 2004; Ibáñez et al., 2004; Itokawa et al., 2013; Konno et al., 2016; Nishioka et al., 2009). Most *SNCA* mutations occur in the N-terminus of the protein α Syn and cause varying degrees of symptoms (Xia et al., 2022). For instance, even within patients with *SNCA*^{A53T} mutations, different symptoms will manifest at different stages of the disease (Markopoulou et al., 2008).

Mutations in *LRRK2* are a common genetic cause of PD, responsible for 3-19% of familial PD and 1-6% of sporadic PD; however these mutations are partially penetrant so it is also among the most common risk factors for PD (Artzi et al., 2017; Latourelle et al., 2008;

Xiong et al., 2017). The most common *LRRK2* mutation, *LRRK2*^{G2019S}, increases its kinase activity and patients with this mutation present similarly to traditional sporadic PD (Kalia et al., 2015; West et al., 2005). Another gene mutated in autosomal dominant PD is *VPS35*, known to cause late-onset PD, and while its direct mechanism of action remains unknown, it is suggested to cause autophagy, lysosomal, and mitochondrial dysfunction (Sassone et al., 2021; Williams et al., 2022). The most recently identified gene implicated in autosomal dominant PD is *RAB32*, specifically the *RAB32* c.213C>G mutation (Ser71Arg), which likely has a similar mechanism of action to *LRRK2*^{G2019S} as *RAB2*^{S71R} leads to an increased LRRK2 kinase activity (Gustavsson et al., 2024).

It is generally thought these autosomal dominant mutations elicit PD through impaired autophagy-lysosomal protein clearance, leading to α Syn accumulation and aggregation, and cellular stress (Dehay et al., 2013; Lill, 2016). Ultimately, these factors lead to dopaminergic neurodegeneration, although the mechanism behind their selective toxicity remains elusive (Sánchez-Danés et al., 2012).

1.3.4 Autosomal recessive PD genes

There are several genes associated with autosomal recessive PD, including some of the more well-known genes *PRKN*, *PINK1*, and *DJI*. Mutations in *PRKN* account for ~50% of all autosomal recessive PD cases and ~15% of sporadic cases when disease onset is prior to 45 years of age (Lücking et al., 2000; Singleton et al., 2013). *PINK1* and *DJI* mutations are more rare, accounting for <10% and <2% of early onset PD cases, respectively (Bonifati, 2012). There are other autosomal recessive genes that cause juvenile onset atypical parkinsonism, including *ATP13A9*, *FBXO7*, and *POLG*, among others (Bonifati, 2012). Autosomal recessive PD genes generally converge on mitochondrial dynamics, which lead to increased reactive oxygen species

and cell death, of which dopaminergic neurons are suggested to be particularly vulnerable (Dias et al., 2013; Lill, 2016).

1.3.5 PD-associated risk genes

Most PD patients do not experience monogenic forms of the disease, instead there are several genes that, when mutated, confer a greater lifetime risk of developing PD. The most common genetic risk factor for PD are mutations in *GBA1* (Sidransky E. et al., 2009). Roughly 5-30% of PD patients and 1-5% of the general population have *GBA1* mutations, depending on the specific population (Stoker et al., 2018; Vieira & Schapira, 2022; Funayama et al., 2023). *GBA1* encodes the protein glucocerebrosidase, which is involved in sphingolipid metabolism and is also affected in Gaucher disease. *GBA1* mutations tend to lead to ER stress and impaired autophagy-lysosomal pathway, in addition to an altered lipid homeostasis, all of which have been shown to increase α Syn accumulation and aggregation (Gegg et al., 2022; Taguchi et al., 2017). What's more, over 90 GWAS loci have been identified and *SNCA* is by far one of the strongest risk loci. (Edwards et al., 2010; The UK Parkinson's Disease Consortium and The Wellcome Trust Case Control Consortium 2, 2011; Nalls et al., 2019; Pedersen et al., 2021; Pan et al., 2023). This has further cemented α Syn in PD whereby it aggregates in disease and is responsible for both monogenic PD and increased risk.

Since the majority of PD cases are sporadic or idiopathic, a multiple hit hypothesis has been proposed, which states that genetic susceptibility (e.g. GWAS risk variant) and environmental exposure (e.g. significant viral infection(s) or head trauma) will act synergistically to increase the individual risk of developing PD at some point in their lifetime (Cabezudo et al., 2020; Hawkes et al., 2007; Patrick et al., 2019; Sulzer, 2007).

1.4 Anatomical and Cellular Pathology in PD

Cellular and molecular hallmarks of PD include dopaminergic neurodegeneration and α Syn aggregation. The cause of dopaminergic neurodegeneration is unknown and there are currently no disease-modifying therapies targeting the degeneration nor protein aggregation.

1.4.1 Dopaminergic neurodegeneration

Dopaminergic neurons in the substantia nigra *pars compacta* (SNc) are the major cell type to degenerate in PD. Typically, a patient will have a 50-80% reduction in striatal dopamine level and SNc dopamine neuron number when overt motor symptoms develop, and the patient is diagnosed with PD (Hirsch et al., 1988; Zarow et al., 2003; Cheng et al., 2010; Fearnley & Lees, 1991; Hornykiewicz, 1998). These dopaminergic neurons in humans produce neuromelanin, a black pigmented substance, making it easier to spot neurodegeneration in post-mortem cases. It is thought that the process of generating neuromelanin and dopamine, which produce reactive oxygen species leading to oxidative stress, confer insurmountable strain on the dopaminergic neurons, leading to their selective vulnerability in disease (Hirsch et al., 1988, 1989; Chocarro et al., 2023). However, the precise reason for the selective vulnerability of dopaminergic neurodegeneration in PD remains elusive. As disease progresses, more brain regions will begin to degenerate in parallel to those to accumulating protein aggregates: LBs and LNs.

1.4.2 α Syn pathology – LBs and LNs

In PD, LBs and LNs accumulate throughout the brain and are mainly composed of the protein α Syn, although recent evidence has found these aggregates to contain additional cellular constituents such as lipids and organelles (Shahmoradian et al., 2019). LBs and LNs have been

found to contain organelles such as mitochondria, autophagosomes, and endolysosomes (Mahul-Mellier et al., 2020). As it stands, the function of these aggregates remains unknown, and the pattern/predictability of aggregates is difficult to decipher. In 2003, the Braak hypothesis was introduced, stating pathological α Syn spreads in a prion-like manner, promoting aggregate and subsequent Lewy pathology in a predictable pattern (Braak et al., 2003). This hypothesis suggested that the topography of α Syn aggregates changes in a predictable pattern consisting of 6 stages, beginning in the dorsal motor nucleus of the vagus nerve or olfactory bulb, spreading throughout regions like the locus coeruleus, SNc, thalamus, and hippocampus, before occupying the cortex in stages 5-6 (Braak et al., 2003). Supporting this hypothesis, α Syn pathology was observed in grafted neurons in post mortem PD brains – fetal ventral mesencephalic transplants were thought to mitigate dopaminergic neuron loss in PD (Kordower et al., 2008; Li et al., 2008). Although this spread of α Syn pathology was not seen in all cases (Mendez et al., 2008). The following year, the α Syn pre-formed fibril model was introduced that relies on α Syn cell-to-cell spreading, and has since become a key tool of PD research (Luk et al., 2009; Luk, Kehm, Carroll, et al., 2012; Luk, Kehm, Zhang, et al., 2012). The Braak hypothesis has since been refined to model the observable bidirectional spread in α Syn pathology, and the intricate differences in α Syn strains that are attributed to the spreading patterns (Borghammer, 2023; Horsager et al., 2020; Just et al., 2022).

It remains unclear exactly how α Syn spreads in this prion-like manner, however its aggregation is dependent on endogenous α Syn (Hijaz & Volpicelli-Daley, 2020; Luk et al., 2012; Vasili et al., 2022). Increasing evidence also supports the level of α Syn protein, post-translational modifications (PTMs), and cellular milieu can also play a role in α Syn aggregation dynamics (Singleton et al., 2003; Nishioka et al., 2006; Flagmeier et al., 2016; Just

et al., 2022). There have been several studies that aim to measure the spread of α Syn pathology, but do not dissect the underlying susceptibility of cells or the topographical measurement of endogenous α Syn used to propagate its aggregation (Caputo et al., 2020; Dadgar-Kiani et al., 2022; Luk et al., 2012; Masuda-Suzukake et al., 2013). Understanding the native topography of α Syn and the mechanism behind cell-specific vulnerability to α Syn aggregates will bring the field closer to fully comprehending the role for α Syn in disease, and PD itself.

Outside of the brain, α Syn pathology can be found in many different tissues, including the retina, gut, spinal cord, and heart (Braak et al., 2006; Böttner et al., 2012; Sumikura et al., 2015; Javanshiri et al., 2022; Raunio et al., 2022; Hart de Ruyter et al., 2023; Wagner et al., 2023). PD is not the only disease to harbor α Syn pathology; there is an overarching disease family termed α -synucleinopathies which encompass diseases including other parkinsonisms, Lewy body dementia, and multiple system atrophy (MSA), among others. For example, in MSA, α Syn pathology is found in oligodendrocytes and these aggregates are called glial cytoplasmic inclusions (Goh et al., 2023; Pfeiffer, 2007). It is thought that cellular milieu contributes to α Syn pathology, and recent work has shown that different aggregation properties of α Syn fibrils depend on their original cell-of-origin (Just et al., 2022; Malfertheiner et al., 2021). It remains unclear precisely why α Syn aggregates or dopaminergic neurons degenerate, however the degree of degeneration – and to a lesser extent aggregation – are correlated to disease severity (Parkinson, 1817; Braak et al., 2003; Hsiao et al., 2014; Takahashi et al., 2019).

1.5 PD diagnosis and therapeutics

Patients are typically diagnosed based on their motor symptoms, their response to dopamine replacement therapies, and their disease progression measured by their symptom presentation. There have been several iterations of clinical rating scales developed over the decades to track the progression of disease and measure therapeutic benefit.

1.5.1 PD diagnosis

In 1967 the Hoehn and Yahr (H&Y) clinical rating scale was introduced to diagnose and measure the progression of PD with 5 stages that increase based on symptom severity (Hoehn & Yahr, 1967). In 1987, the Unified PD Rating Scale (UPDRS) was developed as a flexible scale to monitor PD-related disability and impairment and became widely used (Fahn, 1987). A new rating scale was curated in 2008, headed by the Movement Disorder Society (MDS), to design a more encompassing rating scale, which they named the MDS-UPDRS (Goetz et al., 2008). These scales measure both the motor and non-motor symptoms of PD and account for their severity to track the level of disability as a correlation of disease severity. Clinicians also use dopamine transporter single photon emission computed tomography (DaT-SPECT) scan as a diagnostic tool to measure dopaminergic neurodegeneration via the loss in dopamine transporter signal in the caudoputamen (Bajaj et al., 2013).

1.5.2 Medications

Unfortunately, there are no existing disease modifying therapeutics for PD, only treatments providing symptomatic relief. Many PD treatments target the motor symptoms of disease, with the main target of these treatments to replace or supplement the dopamine that is lost due to dopaminergic neurodegeneration. However, PD symptoms can last for decades, and certain drugs

are not as effective for long-term treatment, therefore medication regimes are often readjusted throughout disease progression.

The most well-known treatment for PD is levodopa, which tends to be used to combat bradykinesia and tremors (Carlsson et al., 1957). Levodopa is a dopamine replacement therapy and is often administered in conjunction with carbidopa to prevent the metabolism of levodopa to dopamine before it can cross the blood-brain barrier (Gandhi & Saadabadi, 2024). Other therapeutics include dopamine agonists, monoamine oxidase B (MAO-B) inhibitors, catechol-O-methyl transferase (COMT) inhibitors, amantadine, anticholinergics, and adenosine A_{2A} receptor antagonists (Cieślak et al., 2008; Fox et al., 2018; *Medications to Treat Parkinson's Disease*, n.d.). Levodopa and dopamine agonists work by replacing (or acting as an agonist to) the dopamine that is lost due to dopaminergic neurodegeneration (Abrams et al., 1971; Koller & Rueda, 1998). MAO-B and COMT inhibitors act by slowing or reducing the premature metabolization of dopamine or its precursor, levodopa (Fabbri et al., 2022; Tan et al., 2022). Amantadine is an NMDA receptor antagonist which promotes the release and reduces the reuptake of dopamine (Bailey & Stone, 1975). Anticholinergics work by counteracting the hyperactivity of cholinergic interneurons in PD, thereby reducing tremors (Katzenschlager et al., 2002; Padilla-Orozco et al., 2022). Adenosine A_{2A} receptor antagonists act by blocking the Adenosine A_{2A} receptors that act antagonistically with dopamine receptors in the striatum, thus enabling more effective dopamine uptake (Cieślak et al., 2008). The non-motor symptoms of PD are generally treated with non-PD-specific therapeutic agents (Batla et al., 2016; Chen & Marsh, 2014; Stacy & Jankovic, 2011; Sun & Armstrong, 2021; Zhuo et al., 2017).

1.5.3 Surgical intervention

There are also different surgical techniques used to combat PD symptoms. The earliest form of surgical intervention for PD was ablation of dysfunctional brain regions, including the thalamus (thalamotomy), globus pallidus (pallidotomy), and subthalamic nucleus (subthalamotomy) to relieve dopamine-related symptoms such as tremor, rigidity, and bradykinesia (Lozano & Lang, 1998; Perry & Lenz, 1998; Alvarez et al., 2005; Chao et al., 2007). Emerging technology has enabled the development of magnetic resonance imaging (MRI)-guided focused ultrasound (MRgFUS) to deliver thermal ablations to these brain regions (Schlesinger et al., 2017; Zaaroor et al., 2017). This technique is favourable as it does not require traditional neurosurgical techniques of opening the skull, thereby reducing the risk of post-operative complications. However, surgical ablation is not used as frequently; typically, clinicians favour deep brain stimulation (DBS). DBS functions similarly to the ablation model by silencing the brain region (thalamus, globus pallidus, or subthalamic nucleus) but uses an implanted electrode at a specific polarity, voltage, pulse duration, and frequency to decrease thalamocortical inhibition, thus increasing motor cortex activation and providing therapeutic benefit to the patient (DeLong, 1990; McGregor & Nelson, 2019; Purves et al., 2001; Volkmann et al., 2006). These parameters can be modified postoperatively to provide longer-lasting relief to the patient for personalized care (Volkmann et al., 2006). The other benefit of DBS over the ablation model is that it is reversible and can be performed bilaterally for more amelioration of symptoms without as much risk for dysarthria, dysphonia, or issues with balance (Bergman et al., 1990; Siegfried & Lippitz, 1994; Benazzouz et al., 1993; Benabid et al., 1995; De Bie et al., 2002; Volkmann, 2004; DeLong & Wichmann, 2012; Mao et al., 2019; Hariz & Blomstedt, 2022).

1.5.4 PD biomarkers

Given the presumed extended prodromal period to PD, there has been a push to identify early biomarkers of disease before overt motor symptom onset, with the goal of developing disease-modifying therapeutics. As the two hallmarks of PD include dopaminergic neurodegeneration and α Syn aggregation, detecting these hallmarks as early as possible have become the target of many biomarkers. For instance, DaT-SPECT scans can be used as a diagnostic tool or to detect early dopaminergic neurodegeneration, since this can occur early in disease progression (Bajaj et al., 2013). Additionally, neurodegeneration and neuroinflammation markers can be detected in cerebrospinal fluid and blood, including markers such as glial fibrillary acid protein (GFAP), neurofilament light chain, amyloid beta, tau, and α Syn, among others (Aamodt et al., 2021; Hall et al., 2012; Kang et al., 2013; Mollenhauer et al., 2011; Ren et al., 2022; Tang et al., 2023). However, some of these neuroinflammatory or neurodysfunction markers are not selective for PD over other neurodegenerative diseases and cannot be used independently to identify early stages of PD. More recently, measuring α Syn aggregation has become an attractive biomarker candidate through the advent of the α Syn seed amplification assay (SAA). The SAA is founded on the prion-like nature of α Syn, whereby aggregate-prone α Syn can seed aggregation when incubated with monomeric, recombinant α Syn *in vitro*. Importantly, the SAA has been validated using patient cerebrospinal fluid to seed aggregation in PD – but not healthy (or non-PD) – cerebrospinal fluid (Höglinger et al., 2024; Simuni et al., 2024). To make this assay more amenable, efforts are focusing on validating this technique from other patient samples including blood, tissue biopsy, and mucosal membrane swabs (Kluge et al., 2022; Gibbons et al., 2023; Okuzumi et al., 2023; Duan et al., 2023). However, this assay has not

yet been approved for use in Canada and therefore a PD diagnosis largely relies on its clinical manifestations.

Other biomarker studies have targeted RBD and olfactory dysfunction in the diagnosis of prodromal PD. According to longitudinal studies, having incidental RBD confers a high (30-91%) risk of developing a neurodegenerative disorder (more specifically, an α -synucleinopathy) within 10 years (Postuma et al., 2010, 2013; Das et al., 2024). Olfactory dysfunction is highly (>90%) prevalent in PD patients, and in two longitudinal studies ~10% of people with olfactory dysfunction were diagnosed with PD within 2-10 years (Ponsen et al., 2004; Haehner et al., 2019; Mollenhauer et al., 2023).

Altogether, developing early biomarkers to detect PD will have a multipronged outcome on disease prognosis, both in its diagnosis and treatment. Accurately detecting or predicting PD earlier in its progression has the potential to unlock disease-modifying therapeutics before a significant amount of dopaminergic neurodegeneration occurs.

1.6 Alpha-synuclein

α Syn is an intrinsically disordered protein encoded by the gene *SNCA* in humans and *Snc*a in rodents. It was first identified in a 1988 study, and named in recognition of its observed synaptic and nuclear localization throughout the central nervous system (Maroteaux et al., 1988). Although α Syn comprises roughly 1% of all cytosolic proteins in the brain, it has been relatively understudied in its native physiology (Bendor et al., 2013; Stefanis, 2012). *SNCA* is a 420 bp gene that encodes a 14 kDa protein with 3 domains: N-terminal, non-amyloid component (NAC), and C-terminal. The N-terminal domain of α Syn spans from residues 1-60, is typically in an

alpha-helical conformation when bound to membranes, and consists of four 11 amino acids repeats (Emamzadeh, 2016; Fakhree et al., 2018). These 11 amino acids contain a highly conserved KTKEGV hexameric repeat which are thought to promote a healthy tetramerization of α Syn to prevent its aggregation (Bartels et al., 2011; Dettmer et al., 2015; Nuber et al., 2018). However, the existence and function of these tetramers, particularly in the context of PD pathogenesis, is somewhat contentious (Burré et al., 2013; Fauvet et al., 2012). Interestingly, most disease-linked mutations in *SNCA* occur within the N-terminal region of α Syn (Koga et al., 2021).

The NAC region was first identified in 1993 where Uéda and colleagues found a peptide in the amyloid deposits of Alzheimer's disease brains (Uéda et al., 1993). Since then, it has been identified as a region of α Syn that spans residues 61-95 and is typically in an alpha-helical conformation when bound to membranes (Bisaglia et al., 2006; Fakhree et al., 2018). As its name suggests, it can form beta-sheet structures and is involved in fibril conformation (Bisaglia et al., 2006; Guerrero-Ferreira et al., 2019; Guzzo et al., 2021).

The C-terminal domain of α Syn spans residues 96-140 and is known as an acidic, unstructured tail that has low hydrophobicity and a high net negative charge (Emamzadeh, 2016). The C-terminus is implicated in many protein-protein interactions and is known to be protective against α Syn aggregation (Park et al., 2002). Interestingly, the most well-documented and disease-associated PTM occurs in the C-terminus of α Syn, specifically the phosphorylation of Ser129 (Fujiwara et al., 2002; Koga et al., 2021).

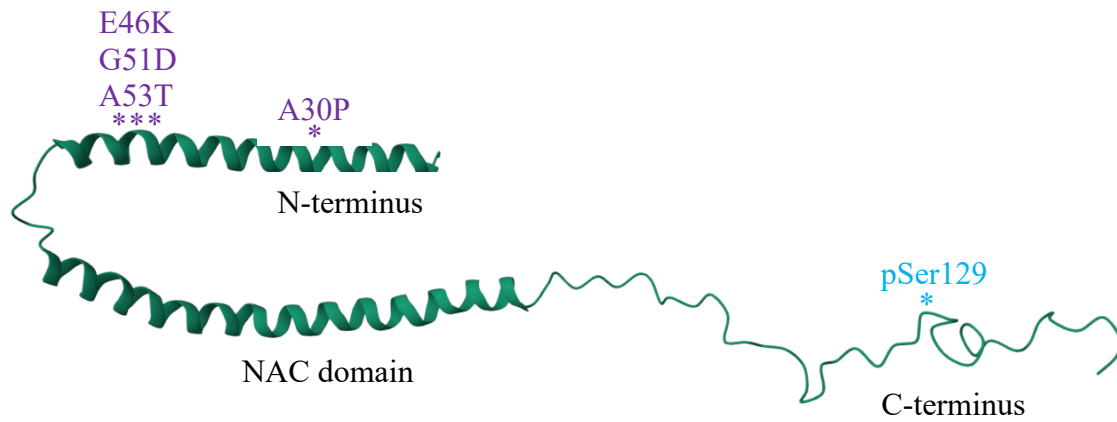


Figure 1.3: Protein structure of alpha-synuclein denoting N-terminus, NAC domain, and C-terminus (adapted from Ulmer et al., 2005). NAC = non amyloid component. Purple denotes mutations associated with Parkinson's disease. Blue denotes phosphorylation site at Serine-129.

1.6.1 Post-translational modifications of α Syn

There are several known PTMs of α Syn with varying degrees of impact on the protein, but most are associated with disease (Barrett & Timothy Greenamyre, 2015; Zhang et al., 2023). Some reports suggest a physiological role of α Syn-pSer129 in reducing its aggregation propensity, and maintaining protein-protein interactions and neurotransmission, however the majority use it as a marker for α Syn pathology in disease (Okochi et al., 2000; Fujiwara et al., 2002; Anderson et al., 2006; Pinho et al., 2019; Ramalingam et al., 2023; Parra-Rivas et al., 2023; Ghanem et al., 2022). α Syn is also known to be phosphorylated at several tyrosines and serines, including Tyr33, Tyr39, Tyr44, Tyr50, Tyr60, Tyr64, Ser87, Tyr125, Tyr133, and Tyr136 with different effects on α Syn dynamics and levels in disease (Chen et al., 2009; Paleologou et al., 2010; Mahul-Mellier et al., 2014; Sano et al., 2021; Sonustun et al., 2022; Zhao et al., 2020; Matsui et al., 2023). There are several kinases that have been implicated in phosphorylating α Syn, including G protein-coupled receptor kinases (GRKs), casein kinase 2 (CK2), polo-like kinase 2 (PLK2), c-Abl, and LRRK2 (Pronin et al., 2000; Ishii et al., 2007; Inglis et al., 2009; Qing, Wong, et al., 2009; Qing, Zhang, et al., 2009; Bergeron et al., 2014; Mahul-Mellier et al., 2014). In addition to phosphorylation, α Syn is also known to be nitrated, acetylated, ubiquitinated, SUMOylated, glycated, and O-GlcNAcylated both natively and in disease (Münch et al., 2000; Tofaris et al., 2003; Nonaka et al., 2005; Shaikh & Nicholson, 2008; Krumova et al., 2011; Trexler & Rhoades, 2012; Fauvet et al., 2012; Burai et al., 2015; Marotta et al., 2015; Rott et al., 2017; Wani et al., 2017; He et al., 2019; Levine et al., 2019; Ryu et al., 2019; Zenko et al., 2023; Balana et al., 2024). Additionally, α Syn can be C-terminally truncated or alternatively

spliced – both of which have been shown to be more aggregate-prone (Baba et al., 1998; Bungeroth et al., 2014; Iyer et al., 2017; Gámez-Valero & Beyer, 2018; Zhang et al., 2022; Hassanzadeh et al., 2023; Röntgen et al., 2024).

1.6.2 Pre-synaptic α Syn

Although α Syn was first described as a nuclear and pre-synaptic protein, most of its native function has been studied based on its pre-synaptic or cytosolic localization. Healthy α Syn exists along a spectrum between a soluble, unstructured state and a membrane-bound, helical state (Burré et al., 2015).

The N-terminus of α Syn can bind to lipids and is known to bind to presynaptic vesicles to mediate synaptic function (Jo et al., 2000). It is this binding of lipid rafts that aid in its pre-synaptic localization (Fortin et al., 2004; Zabrocki et al., 2008). Depending on the size and curvature of the vesicle, α Syn can adopt different conformations including an elongated or broken α -helix for low curvature membranes larger than 100 nm or highly curved membranes smaller than 40 nm, respectively (Burré et al., 2018). The C-terminus of α Syn can interact with vesicles, and has been shown to do this in a calcium-dependent manner (Lautenschläger et al., 2018).

To better understand the role of α Syn, both in healthy and disease states, several groups have aimed to identify protein interactors of α Syn. In healthy states, many α Syn interacting proteins are primarily presynaptic. The C-terminus of α Syn was found to bind to synaptobrevin-2 (Syb2)/vesicular associated membrane protein 2 (VAMP2) and regulate soluble *N*-ethylmaleimide-sensitive fusion (NSF) attachment protein receptor (SNARE) complex assembly (Burré et al., 2010). Further research investigating this interaction revealed a role for

α Syn in vesicle recycling and found it to regulate synaptic vesicle endocytosis (Vargas et al., 2014; Burré, 2015; Sun et al., 2019). However, α Syn disperses from the synapse following neuronal activity, highlighting its weak interaction with synaptic vesicles (Fortin et al., 2005).

Interestingly, α Syn knockout mice are relatively normal under baseline conditions, exhibiting significant impairments in synaptic response to prolonged stimulation (Murphy et al., 2000; Cabin et al., 2002). Cabin et al suggest that α Syn is required for the genesis and/or maintenance of synaptic vesicles in the ‘reserve’ or ‘resting’ pools, and results in synaptic deficiencies upon depletion of docked vesicles (Cabin et al., 2002). This is likely due to the interaction between α Syn and synapsins (Atias et al., 2019; Stavsky et al., 2023). Similarly, α Syn overexpression models found a suppression of exocytosis and an opposite effect in knockouts (Abeliovich et al., 2000; Garcia-Reitböck et al., 2010; Greten-Harrison et al., 2010; Anwar et al., 2011). α Syn is also known to interact with proteins involved in membrane trafficking and protein transport (Chung et al., 2017). α Syn can also interact with the SNARE complex in disease states. Upon cell stress, α Syn binds with and inactivates the SNARE protein Ykt6 to prevent lysosomal protein degradation, thereby promoting α Syn accumulation and aggregation (Cuddy et al., 2019).

In disease, α Syn mislocalizes from the presynaptic space, which can lead to novel disease-related interacting proteins. One group identified α Syn-pSer129/Tyr125-specific binding partners of α Syn using immunoprecipitation coupled with mass spectrometry (McFarland et al., 2008). Here, they identified α Syn-pSer129/Tyr125-specific interactors including microtubule-associated protein 1B (MAP1B), β - and γ -actin, non-muscle myosins, and neurofilament triplet L protein (NEFL), among others. They also identified many mitochondrial

electron transport chain subunits enriched in the non-phosphorylated α Syn interactome. Another group built on identifying the α Syn interactome using ascorbate peroxidase (APEX) to label proximal proteins followed by immunoprecipitation and mass spectrometry (Chung et al., 2017). Similarly, they identified cytoskeleton, endocytosis, and trafficking proteins. Moreover, they identified proteins involved in mRNA metabolism, including Pabpc1, G3bp1/2, and Tia1/Tial1. Indeed, α Syn was further implicated in mRNA metabolism by the same group, where it was found to bind to Edc4, involved in decapping mRNA at P-bodies (Hallaceli et al., 2022). They further showed that α Syn upregulation can disrupt Edc4 complexes and alter mRNA metabolism, suggesting a possible role in disease.

The basic α Syn interactome can also be compared and combined with other neurodegenerative-associated proteins, including in a more recent study that compared α Syn and tau interactomes (Griffin et al., 2023). This group compared the interactomes following fibril treatment, seeding pathology of α Syn and tau, along with disease-associated mutations including α Syn^{A53T} and tau^{P301L}. They found similar interacting proteins between α Syn, tau, and previous interactome studies, and that these interactors are diminished upon fibril treatment. Altogether, these interactome studies highlight the nuance of protein-protein interactions in neurodegenerative diseases, and open alternative targets for disease-modifying therapeutics.

1.6.3 α Syn localization to organelles

α Syn is known to localize to various organelles, both in healthy and disease states. α Syn has an affinity for binding lipid membranes, and has been noted to associate with membranes of the endoplasmic reticulum (ER)/Golgi, mitochondria, and endo/lysosomes (Miraglia et al., 2018).

In α Syn transgenic mouse models and cell lines, α Syn was found to bind to the luminal side of the ER, is associated with ER trafficking, and is known to cause ER stress upon aggregation (Colla et al., 2012; Colla, 2019). Studies in yeast found that α Syn accumulation negatively impacted ER-Golgi vesicular trafficking and led to dopaminergic neurodegeneration in animal models (Cooper et al., 2006; Gitler et al., 2008).

Mitochondrial dysfunction has been a focus in PD research, however the role of α Syn in mitochondrial dysfunction remains elusive. It is thought that α Syn has a cryptic mitochondrial targeting sequence due to the positively charged lysines in the N-terminal region (Li et al., 2007; Devi et al., 2008; Nakamura et al., 2008). Importantly, α Syn associates with the inner mitochondrial membrane to elicit toxic effects, including impaired complex I activity and increased reactive oxygen species, contributing to disease (Devi et al., 2008). It has been shown that mutations in the N-terminus of α Syn affect its mitochondrial localization, due to its decreased lipid binding affinity, leading to mitochondrial dysfunction (Pozo Devoto et al., 2017). Moreover, α Syn has been shown to bind to the outer mitochondrial membrane through its interaction with cardiolipin protein, which becomes externalized in disease (Lurette et al., 2023; Ryan et al., 2018). Externalized cardiolipin can promote the “healthy” folding of α Syn into an α -helical state, however prolonged cardiolipin externalization and α Syn aggregation lead to mitochondrial fragmentation and degradation (Lurette et al., 2023; Ryan et al., 2018).

The main pathway responsible for α Syn degradation is the endolysosomal pathway, which is disrupted in PD (Vidyadhara et al., 2019). Similar to the ER/Golgi and mitochondria, α Syn has been shown to colocalize with endosomes and lysosomes (Bourdenx et al., 2014). This interaction has been a target for interventional therapies for PD. A recent paper used a peptide

inhibitor to disrupt the interaction between α Syn and a component of the endosomal sorting complex required for transport III (ESCRT-III), to rescue endolysosomal activity and reduce dopaminergic neurodegeneration (Nim et al., 2023). This has implications not only for idiopathic PD, but also for patients with genetic mutations that affect the endolysosomal pathway, including those with *GBA1* and *LRRK2* mutations, among others (Yahya et al., 2023).

1.6.4 Native nuclear α Syn

Early evidence has shown a small population of nuclear α Syn in healthy conditions has important physiological roles (Goers et al., 2003; Maroteaux et al., 1988; Schaser et al., 2019). Interestingly, α Syn phosphorylation at Ser129 is typically thought to be toxic, however there is evidence to support that α Syn-pSer129 contributes to the nuclear localization of α Syn, both of which were shown to be neuroprotective (Pinho, Paiva, Jerčić, et al., 2019). Further investigation into its role in the nucleus has revealed that α Syn can bind to DNA and histones to modulate gene expression and DNA repair (Siddiqui et al., 2012; Paiva et al., 2018; Jiang et al., 2018; Schaser et al., 2019). One group found that α Syn forms a tight complex with histones and that histones accelerate the rate of α Syn fibrilization (Goers et al., 2003). Using chromatin immunoprecipitation techniques, α Syn has been shown to bind to the promoter region of *PGC1 α* and alter downstream mRNA and protein levels of *PGC1 α* -target genes upon oxidative stress (Siddiqui et al., 2012). Another group found that α Syn^{A30P} has an increased propensity to bind to DNA and that this yielded an upregulation in *COL4A2*, leading to increased endoplasmic reticulum stress and Golgi fragmentation in dopaminergic neurons (Paiva et al., 2018). Davidi et al suggest that α Syn is imported into the nucleus through binding to retinoic acid, where α Syn can then selectively enhance gene transcription (Davidi et al., 2020). Broadly, evidence supports

α Syn binding preferentially to GC-rich genomic regions and can alter DNA conformations (Vasudevaraju et al., 2012). α Syn also colocalizes with DNA damage response components, including phosphorylated histone 2 A.X and poly-ADP ribose polymer (Schaser et al., 2019). They also found that α Syn not only binds to double stranded DNA at damage sites, but facilitates DNA non-homologous end-joining for DNA repair (Rose et al., 2024; Schaser et al., 2019). Interestingly, DNA double stranded breaks increase in α Syn knockout models, further supporting the role of α Syn in DNA damage repair (Schaser et al., 2019). Recently, this group also found that α Syn localizes to the nucleolus to facilitate ribosomal DNA double stranded break repair in melanoma (Arnold et al., 2024). This is an important finding due to the positive correlation between melanoma and PD (Ong et al., 2014; Skibba et al., 1972). Moreover, it highlights a native, healthy role for nuclear α Syn that may be disrupted in disease upon nuclear accumulation.

1.6.5 Neurotoxic nuclear localization

Although native nuclear α Syn has been suggested to be neuroprotective, the accumulation of nuclear α Syn can lead to significant neurotoxicity. This neurotoxicity has been shown in several models, but the mechanisms of toxicity remain unknown. There is a documented increase in nuclear α Syn from diseased post-mortem brain samples quantified through both immunofluorescence and cell fractionation studies (Koss et al., 2022; Rousseaux et al., 2016). Incidental studies have also found several insults that lead to an increase in nuclear α Syn, including overexpressing α Syn and other proteins, α Syn mutations and PTMs, and some known environmental exposures linked to PD (Cannon et al., 2013; Chen et al., 2020; Fares et

al., 2014; Kontopoulos et al., 2006; Manning-Bog et al., 2002; Masliah et al., 2000; Pinho et al., 2019; Rousseaux et al., 2016; Schell et al., 2009; Sousa et al., 2009).

Although α Syn does not have a canonical nuclear localization signal, studies have shown that mutating α Syn at A30P, E46K, G51D, and A53T, known familial PD mutations, leads to increased nuclear α Syn (Cannon et al., 2013; Chen et al., 2020; Fares et al., 2014; Kontopoulos et al., 2006; Sousa et al., 2009). Additionally, phosphorylation at Ser129 is promoted upon α Syn nuclear accumulation (Pan et al., 2022; Pinho et al., 2019). Early studies generating mouse lines that overexpress human α Syn in rodent models also noted an increase in nuclear α Syn (Masliah et al., 2000).

While α Syn is small enough to passively diffuse in and out of the nucleus, some studies have shown that overexpressing certain proteins can lead to the accumulation of nuclear α Syn, whether by increasing its nuclear trafficking or stabilizing α Syn within the nucleus. Rousseaux et al performed a knockdown screen to explore druggable targets that may impact α Syn protein levels. This study found tripartite motif-containing 28 (TRIM28) overexpression led to α Syn stabilization and an increase in its nuclear accumulation (Rousseaux et al., 2016). Davidi et al profiled transcriptional changes upon α Syn overexpression and found altered regulation of retinoic acid receptors. When they exposed different cell lines and cultured murine neurons to retinoic acid, they noted an increase in nuclear α Syn localization, which they later discovered was due to α Syn binding directly to retinoic acid prior to its nuclear import (Davidi et al., 2020).

Although several research groups have used α Syn mutation, knock-in, or overexpression models to study the consequences of nuclear α Syn accumulation, none of these pinpoint the cause of nuclear α Syn accumulation in disease. However, some environmental exposure models

have shown increased nuclear α Syn, among other phenotypes. Understanding why and how α Syn accumulates in the nucleus in disease, in addition to what it is doing in the nucleus upon accumulation, will be imperative in developing better PD therapeutics.

1.7 Preclinical models of PD

Several models have been developed to study varying aspects of PD. Some of these include exploiting genetic manipulations (of both *SNCA* and other PD-associated genes), environmental toxins (a growing list of toxins, including paraquat and rotenone), α Syn pre-formed fibrils (PFFs), or a combination thereof. Some of these models, particularly the environmental exposure models, are better at depicting the dopaminergic neurodegeneration aspect of PD, including paraquat, rotenone, permethrin, 6-hydroxydopamine (6-OHDA), and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment (Ungerstedt, 1968; Kopin & Markey, 1988; McCormack et al., 2005; Cannon et al., 2009; Nasuti et al., 2007; Tanner et al., 2011; Goldman, 2014). Whereas others model α Syn accumulation and aggregation, including α Syn mutation and overexpression, rotenone exposure, and α Syn PFFs (Masliah et al., 2000; Cannon et al., 2009; Luk et al., 2012). Other models explore known genetic forms of PD to decipher the molecular mechanisms at play (Chandran et al., 2008; Xiong et al., 2017; Do et al., 2021). While there is no current model that perfectly encompasses all clinicopathological features of PD, these models have led to significant progress in our understanding of disease.

1.7.1 Invertebrate models of nuclear α Syn

Invertebrate models of PD are useful for screening experiments to understand the dynamics of α Syn in a quick and efficient manner. A frequently used invertebrate model in PD

research is the *Drosophila* to study dopaminergic neurodegeneration, aberrant α Syn throughout the brain, and behavioural deficits (Feany & Bender, 2000). A key study from 2006 used *Drosophila* to overexpress α Syn with a nuclear localization sequence (NLS) or nuclear export sequence (NES) tag. Following nuclear α Syn overexpression (α Syn-NLS), they noted significant dopaminergic neurodegeneration, which was in stark contrast to the α Syn-NES overexpression that showed nearly no degeneration (Kontopoulos et al., 2006). They also used this model to show increased nuclear abundance of α Syn with the familial mutations α Syn^{A30P} and α Syn^{A53T}.

1.7.2 Murine models of nuclear α Syn

While invertebrate models provide useful screening tools, rodent models of PD have tremendous benefit in providing more similar anatomy, physiology, and genetics to humans, enabling more translation from model to patient. One model simply overexpressing human α Syn had found an increase in nuclear α Syn, although this model is not typically used to directly target nuclear α Syn (Masliah et al., 2000). Some familial α Syn mutations also lead to an increase in nuclear α Syn, which has been exemplified in mouse models of these mutations, however these too are not typically used to directly study the consequences of nuclear α Syn (Kahle et al., 2000; Fares et al., 2014; Paiva et al., 2018). To directly study nuclear α Syn, researchers have used several different models.

One model uses paraquat, a known environmental exposure that increases an individual's risk of developing PD, to cause an upregulation and aggregation of α Syn and its nuclear mislocalization (Goers et al., 2003). This model was used to describe how α Syn can bind to histones. However, it is more common to study nuclear α Syn using genetic models. One group has used stereotaxic injection of adeno-associated virus (AAV) to overexpress α Syn with an NLS

or NES tag to study the mechanism and consequence of nuclear α Syn accumulation (Ma et al., 2014; Pan et al., 2022, 2023). Using this model, they corroborated the *Drosophila* studies showing α Syn-NLS overexpression leads to more dopaminergic neurodegeneration than α Syn overexpression alone, and that α Syn-NES overexpression was comparable to healthy controls (Ma et al., 2014). They went on to find that α Syn-NLS overexpression induced memory and motor impairments in mice and suggested this was due to excess DNA damage and decreased hippocampal neurogenesis (Pan et al., 2022, 2023).

Conversely, there is evidence to support a healthy role for nuclear α Syn. For this, researchers have used *Snca* knockout mice to study the effects of reduced nuclear α Syn. These studies have found that α Syn is involved in double stranded DNA break repair, whereby *Snca* knockout mice have increased double-stranded DNA damage that can be rescued by overexpressing α Syn, and that α Syn colocalizes with other proteins involved in DNA double-stranded break repair (Arnold et al., 2024; Schaser et al., 2019).

1.8 Statement of research problem, rationale, and objectives

PD is a multifactorial disease and remains poorly understood. Fundamental research targeting the underlying mechanisms of disease in addition to pre-clinical research to understand potential targets of therapeutic intervention are crucial to combat this highly prevalent neurodegenerative disease. Patient prognosis is typically dependent on the time at which treatment begins; therefore, targeting an earlier timepoint of disease progression is imperative. This has also been made easier with the validation of early biomarkers of disease. Studying an earlier marker of pathology, the mislocalization of α Syn in the nucleus, will provide better

insight into the initiation and progression of disease. Additionally, understanding the cells that may be vulnerable to altered α Syn dynamics, including mislocalization and aggregation, is an important step to exposing more of the etiology of PD.

To gain insight into the underlying mechanisms of PD and to better study the nuclear accumulation of α Syn, we began by generating and characterizing a mouse (*Snca*^{NLS}) with an NLS knocked-in to the endogenous *Snca* locus, so as not to rely on its overexpression. This enabled the exploration of the consequences of chronic nuclear accumulation of α Syn in both heterozygous and homozygous mice without confounding effects due to its overexpression. In chapter 1, I detail the characterization of this novel mouse model that yielded significant age-dependent motor deficits, reduced survival, and cortical neurodegeneration in the absence of disease-associated α Syn Ser129 phosphorylation and aggregation. This is an important distinction as we can directly attribute these phenotypes to the toxic gain-of-function of nuclear α Syn.

The next chapter aimed to identify possible protein-protein interactions that could be driving the observed phenotypes. By performing TurboID, a proximity proteomics tool, to label proteins within proximity to, or in complex with nuclear α Syn in neurons, we can identify proteins that could be aberrantly interacting with α Syn in disease. Indeed, we identified several proteins that were found to interact or exist within proximity of nuclear α Syn. One protein to note, Luc71, was found to preferentially interact with nuclear α Syn. Although Luc71 has not been previously studied in the context of PD, it is known to be involved in RNA binding and splicing, which are also known to be affected in PD and could be mediated by nuclear α Syn-Luc71 interaction (Tufarelli et al., 2001; La Cognata et al., 2015). We also noted increased α Syn

abundance following Luc7l overexpression, which could suggest a possible stabilization role for Luc7l in disease, contributing to α Syn accumulation and aggregation.

In addition to understanding why and how nuclear α Syn can elicit its toxicity; it is also important to understand how α Syn can spread from cell-to-cell. To gain more insight into the spreading trajectory of α Syn, we must first identify the native topography of α Syn as a base to map aggregation dynamics. Due to its mainly presynaptic and cytosolic localization, measuring its protein topography is difficult. Recent single-cell sequencing advances or *in situ* fluorescent imaging techniques can be used to determine which cells express *Snc*a, however this does not necessarily inform protein localization. To overcome these issues, we repurposed the *Snc*a^{NLS} mouse to map α Syn⁺ cells throughout key regions that are affected in PD, including the brain, spinal cord, retina, and gut. We found a high degree of correlation between α Syn protein and *Snc*a RNA and noted α Syn was primarily neuronal. However, α Syn was not present or expressed to the same degree in all neurons. For example, α Syn intensity was strong in ChAT⁺ neurons in the dorsal motor nucleus of the vagus but was not detectable in ChAT⁺ neurons in the spinal cord. Additionally, we noted many cells in the retina and all neurons in the gut express some degree of α Syn, although to different extents. This atlas provides a framework upon which further research can be performed to explore the specific vulnerability of these cells and whether the cell-specific dosage of α Syn contributes to this vulnerability.

Altogether, we hypothesize that the constitutive nuclear accumulation of α Syn is neurotoxic and contributes to PD, which may be mediated through α Syn interaction with specific nuclear proteins including Luc7l. Additionally, mapping α Syn topography throughout the central

nervous system and the gut has the potential to provide insight into the cell-specific vulnerability of α Syn pathology in PD.

Overall objective: Understand the neurotoxic mechanisms of nuclear α Syn in disease and isolate cells that may be particularly vulnerable to changes in α Syn metabolism.

Specific objectives:

Objective 1: Model the chronic nuclear accumulation of α Syn in a novel mouse to determine phenotypes that are specifically due to its nuclear accumulation without confounding overexpression.

Objective 2: Determine potential protein interactors with nuclear α Syn that may be contributing to disease to highlight future research avenues for targeted therapeutics.

Objective 3: Map the topography of α Syn throughout the mouse central nervous system and gut to gain insight on native α Syn localization.

The three objectives stated above are addressed in the following chapters 2, 3, and 4, respectively.

Chapter 2: Constitutive nuclear accumulation of endogenous alpha-synuclein in mice causes motor impairment and cortical dysfunction, independent of protein aggregation

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Rousseaux MWC

Statement of author contribution

This manuscript details the characterization of the novel *Snca*^{NLS} mouse, which mislocalizes endogenous α Syn to the nucleus via the knock-in of a nuclear localization sequence (NLS). We found that these mice display age-dependent motor deficits in multiple behaviour assays in addition to cortical thinning specific to layer V/VI of the motor cortex. We performed mass spectrometry on the cortex and found significant protein dysregulation in the *Snca*^{NLS} mice relative to controls. We also note that these phenotypes were not driven by α Syn aggregation, phosphorylation of Ser129, nor loss of pre-synaptic α Syn, suggesting a toxic nuclear gain-of-function.

Haley Geertsma and Dr. Maxime Rousseaux conceptualized the study. **Haley Geertsma** generated and maintained mouse cohorts, performed all behavioural tasks (with help from Dr. Konrad Ricke, Zoe Fisk, Jean-Louis Parmasad, and Steve Callaghan), performed most histological and biochemical assays, and imaged and analyzed most figures in this article. Terry Suk obtained all confocal images, performed qPCR experiments, and provided significant intellectual support. Dr. Konrad Ricke aided in tissue acquisition/processing and provided significant intellectual support. Kyra Horsthuis aided in the analysis of stained tissue. Jean-Louis Parmasad and Terry Suk harvested and maintained all primary cortical neurons. Dr. Maxime Rousseaux designed the knock-in mice and assisted in data collection and analysis. **Haley Geertsma** and Dr. Maxime Rousseaux wrote the manuscript and all authors provided edits.

Constitutive nuclear accumulation of endogenous alpha-synuclein in mice causes motor impairment and cortical dysfunction, independent of protein aggregation.

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Abstract

A growing body of evidence suggests that nuclear alpha-synuclein (α Syn) plays a role in the pathogenesis of Parkinson's disease (PD). However, this question has been difficult to address as controlling the localization of α Syn in experimental systems often requires protein overexpression, which affects its aggregation propensity. To overcome this, we engineered *Snca*^{NLS} mice which localize endogenous α Syn to the nucleus. We characterized these mice on a behavioral, histological, and biochemical level to determine whether the increase of nuclear α Syn is sufficient to elicit PD-like phenotypes. *Snca*^{NLS} mice exhibit age-dependent motor deficits and altered gastrointestinal function. We found that these phenotypes were not linked to α Syn aggregation or phosphorylation. Through histological analyses, we observed motor cortex atrophy in the absence of midbrain dopaminergic neurodegeneration. We sampled cortical proteomes of *Snca*^{NLS} mice and controls to determine the molecular underpinnings of these pathologies. Interestingly, we found several dysregulated proteins involved in dopaminergic signalling, including Darpp32, Pde10a, and Gng7, which we further confirmed was decreased in cortical samples of the *Snca*^{NLS} mice compared to controls. These results suggest that chronic *endogenous* nuclear α Syn can elicit toxic phenotypes in mice, independent of its aggregation. This model raises key questions related to the mechanism of α Syn toxicity in PD and provides a new model to study an underappreciated aspect of PD pathogenesis.

Introduction

Alpha synuclein (α Syn) is a protein notorious for its involvement in Parkinson's disease (PD) pathogenesis. For one, it is a primary constituent of Lewy bodies and Lewy neurites, pathological hallmarks of PD (Spillantini et al., 1997). Moreover, copy number variations and missense mutations in the α Syn gene, *SNCA*, cause genetic forms of PD, further reinforcing its involvement in disease etiology (Polymeropoulos et al., 1997; Krüger et al., 1998; Zarranz et al., 2004; Appel-Cresswell et al., 2013; Fares et al., 2014; Ghosh et al., 2014; Konno et al., 2016). α Syn was first described as a presynaptic and nuclear protein (Maroteaux et al., 1988). However, nuclear α Syn has largely been overshadowed by a focus on its cytoplasmic/synaptic form, likely due to the cytoplasmic localization of Lewy bodies. Despite this, several studies have linked nuclear α Syn to PD on multiple levels: in cell (Herman & Coffin, 1986; Kahle, Neumann, Ozmen, Muller, et al., 2000; Specht et al., 2005; Jiang et al., 2016; Chen et al., 2020) and animal (Fares et al., 2014; Masliah et al., 2000; Goers et al., 2003; Kontopoulos et al., 2006; Huang et al., 2011) models of PD, as well as in brain tissue from individuals with α Syn pathologies (synucleinopathy; Koss et al., 2021; Pinho, Paiva, Jercic, et al., 2019; Rousseaux et al., 2016). These studies examined the role of nuclear α Syn by overexpressing it together with mutations or a nuclear localization signal (NLS; Specht et al., 2005; Masliah et al., 2000; Kontopoulos et al., 2006; Huang et al., 2011; McLean et al., 2000; Pinho, Paiva, Jercic, et al., 2019), or upon toxin exposure (Goers et al., 2003), hinting at a role for nuclear α Syn in disease pathogenesis by its involvement in DNA binding (Ciron et al., 2015; Paiva et al., 2018) or histone modification (Kontopoulos et al., 2006) to alter transcription, and in DNA repair (Schaser et al., 2019). While these studies support a link between nuclear α Syn and PD, its specific role in disease – whether deleterious or beneficial – remains clouded due to the reliance on its overexpression or

exogenous stressors, making it difficult to parse out the driver of toxicity in the absence of α Syn aggregation.

To directly test the consequence of chronic α Syn mislocalization to the nucleus *in vivo*, without resorting to protein overexpression, we engineered a mouse model to endogenously express α Syn with a C-terminal NLS-Flag tag driving its mislocalization to the nucleus. We extensively characterized these mice at the behavioral, histological, and biochemical level to assess whether chronic nuclear localization of α Syn causes age-dependent phenotypes resembling PD or related α Syn proteinopathies.

Results

Effective nuclear targeting of α Syn in *Snc*^{NLS} mice

To study if nuclear α Syn is sufficient to elicit age-related behavioral and pathological phenotypes, we generated a mouse line that targets endogenous α Syn to the nucleus via the knockin of an NLS-Flag tag on α Syn (*Snc*^{NLS}). The NLS-Flag construct was targeted to the 3' end of the *Snc* coding sequence with the modified *Snc*-NLS-Flag gene predicted to transcribe a fusion protein of wildtype α Syn with a C-terminal NLS-Flag tag (**Fig. 1A**). After generating and backcrossing mice (see Materials and Methods and **Supplementary Figure 1**), we confirmed the knockin via sequencing (**Supplementary Figure 1B**) and were able to distinguish between the genotypes via a PCR band shift (**Fig. 1B**) and a larger protein size via western blot (**Fig. 1C**). Mice were born at expected Mendelian ratios (**Supplementary Figure 1C**), confirming that insertion of this tag did not pose major developmental deficits.

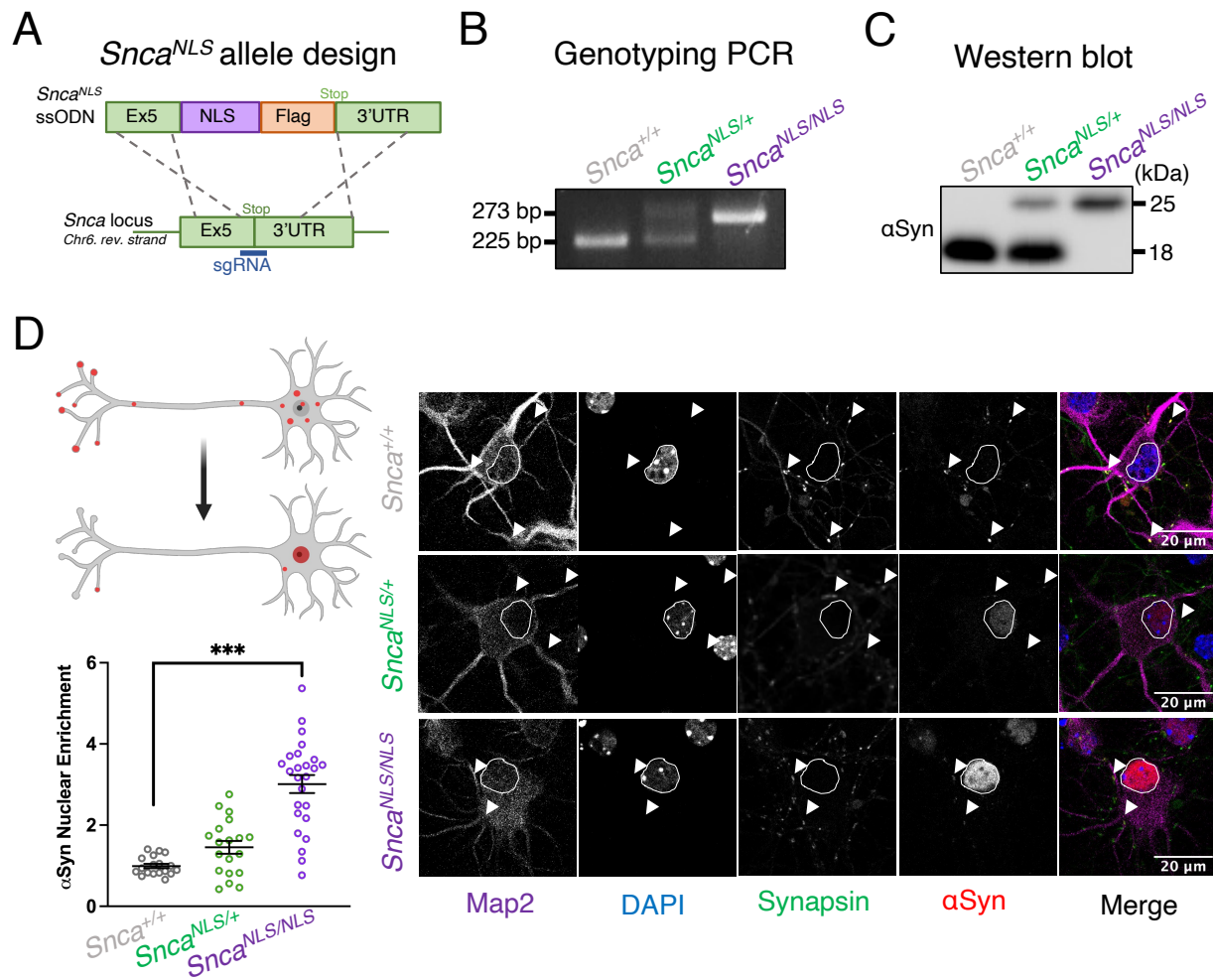


Figure 1: *Snca*^{NLS} mice effectively target αSyn to the nucleus *in vitro* and *in vivo*. (A) *Snca*^{NLS-Flag} knock-in scheme with C-terminal NLS-Flag tag. Visualization of the knockin via (B) PCR and (C) western blot. (D) Illustration of nuclear localization of αSyn (upper left) with quantification (lower left) of nuclear αSyn from primary cortical neurons in wildtype (top right panels), *Snca*^{NLS/+} (middle right panels) and *Snca*^{NLS/NLS} (bottom right panels; *n* = 3). Protein quantification (lower left) depicts nested relative per-cell intensity of nuclear αSyn from three independent experiments. White arrows denote presynaptic αSyn and nuclei are circled in white. Nested one-way ANOVA with Bonferroni multiple comparison: ** denotes *P* < 0.001.

To examine the efficiency of the NLS-Flag tag, we cultured primary cortical neurons for 7 days *in vitro* and quantified the level of nuclear α Syn through immunofluorescent microscopy. We observed a 3-fold increase in nuclear α Syn in *Snca*^{NLS/NLS} and a 1.5-fold increase in *Snca*^{NLS/+} compared to *Snca*^{+/+} (wildtype) cells (**Fig. 1D**). This trend was consistent in stained adult mouse brain tissue at both 2-months and 18-months where we found a 2-5-fold increase in nuclear α Syn in the cortex (**Fig. 2A**), dentate gyrus (**Fig. 2B**), and substantia nigra (**Fig. 2C**). Importantly, this roughly corresponds to the 2.5-3-fold increase of nuclear α Syn which we and others have previously observed in post-mortem brain tissue from individuals with PD or other animal models of synucleinopathy, suggesting the model displays a disease-relevant increase of nuclear α Syn (Fares et al., 2014; Masliah et al., 2000; Goers et al., 2003; Kontopoulos et al., 2006; Huang et al., 2011; Rousseaux et al., 2016).

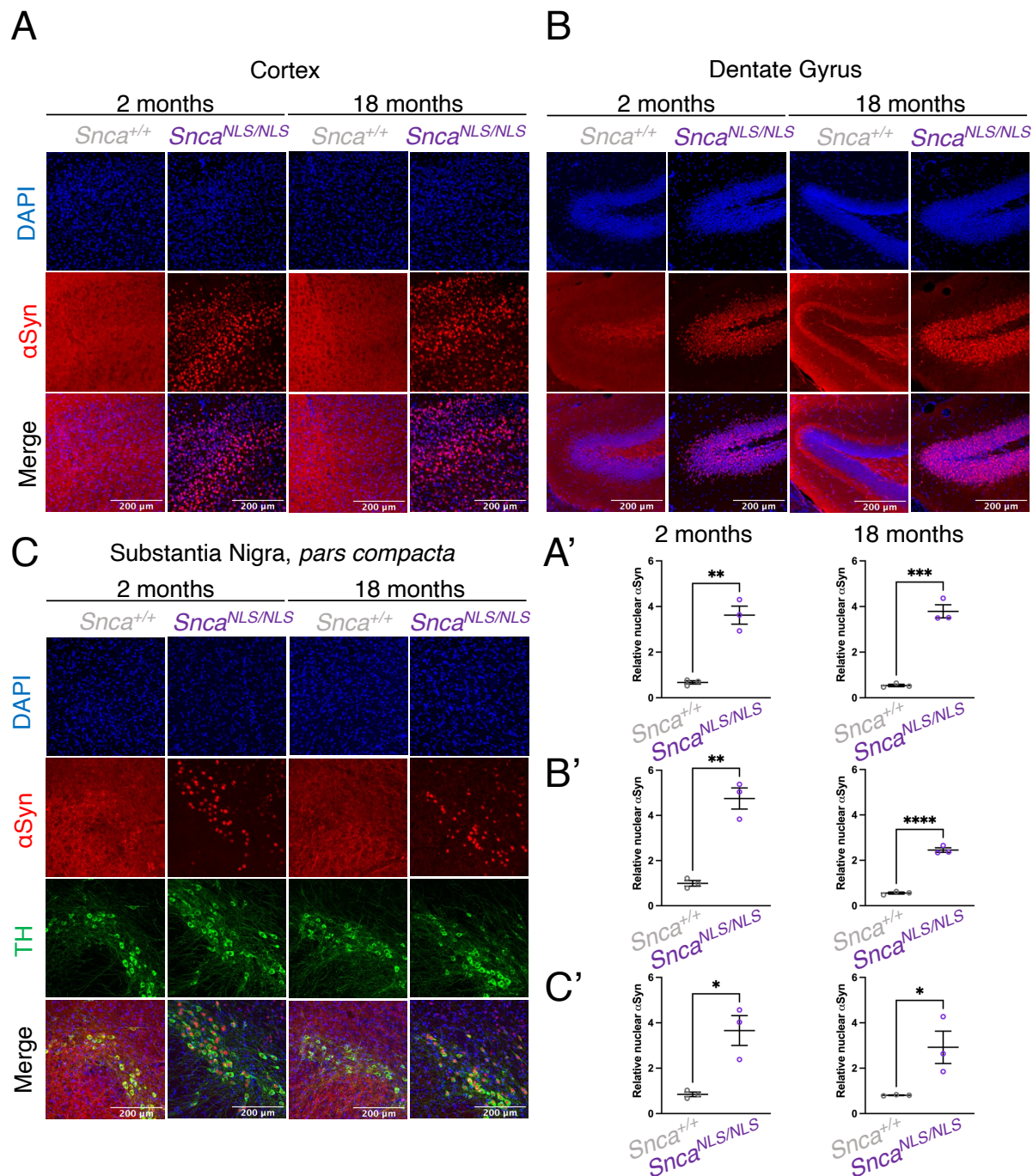


Figure 2: *Snca*^{NLS} mice exhibit a consistent 2–5-fold increase in nuclear α Syn with age. Immunofluorescent images of 2- (left) and 18-month (right) mouse (A) cortex, (B) dentate gyrus and (C) substantia nigra, *pars compacta*. Nuclear enrichment of α Syn was quantified for (A'–C') all brain regions at both 2- (left) and 18-months (right). Unpaired, two-tailed *t*-test: *, **, ***, **** denote $P < 0.05$, < 0.01 , < 0.001 and < 0.0001 , respectively.

Increased nuclear α Syn leads to an age-dependent motor decline

To test whether chronic nuclear accumulation of α Syn is sufficient to elicit PD-like phenotypes over time, we subjected *Snca*^{NLS/NLS} mice and littermates to a battery of behavior tests at 3-, 9-, and 18-months of age. We found that the *Snca*^{NLS/+} and *Snca*^{NLS/NLS} mice performed similarly to wildtype at 3-months of age (**Fig. 3A**). By 9-months, however, the *Snca*^{NLS/NLS} mice displayed a significant motor deficit in rotarod (**Fig. 3B**) as well as a delayed time to contact their forepaws in the adhesive test (**Fig. 3C**). Interestingly, *Snca*^{NLS/+} mice also exhibited a significant deficit on the rotarod test – suggestive of a dominant phenotype. This rotarod deficit was not observed when testing *Snca*^{-/-} mice at 9-months (**Supplementary Figure 2**), suggesting it is due to the accumulation of nuclear α Syn and not a loss-of-function of pre-synaptic α Syn. Surprisingly, after aging the *Snca*^{NLS} mice to 18 months, we observed milder motor phenotypes relative to wildtype controls; likely due to the 18-month wildtype mice showing increased difficulty at performing these tasks when comparing their motor ability to that at 3- and 9-months of age (**Supplementary Figure 3**).

With increasing awareness around non-motor symptoms in PD, we also measured cognition, anxiety, and overall wellness in the *Snca*^{NLS} line. We found that *Snca*^{NLS/NLS} and *Snca*^{NLS/+} mice performed similarly to their wildtype littermates in non-motor behavior tests at all timepoints (**Supplementary Figures 4-6**). In addition to motor decline, people living with PD often experience gastrointestinal difficulties such as constipation ([Cheon et al., 2008](#); [Siddiqui et al., 2002](#)). To measure constipation in our mice, we examined fecal excretions in the span of an hour. We found that 18-month-old *Snca*^{NLS/NLS} mice excretions contained significantly less water than their wildtype counterparts (**Fig. 3D**). Lastly, we observed a trend for early lethality in the *Snca*^{NLS/NLS} mice, whereby 37% of *Snca*^{NLS/NLS} mice died by 20 months of age, compared to 14%

of wildtype littermates ($p=0.0637$, **Fig. 3E**). Taken together, the *Snc*^{NLS/NLS} mice display age-dependent motor and gastrointestinal dysfunction reminiscent of PD.

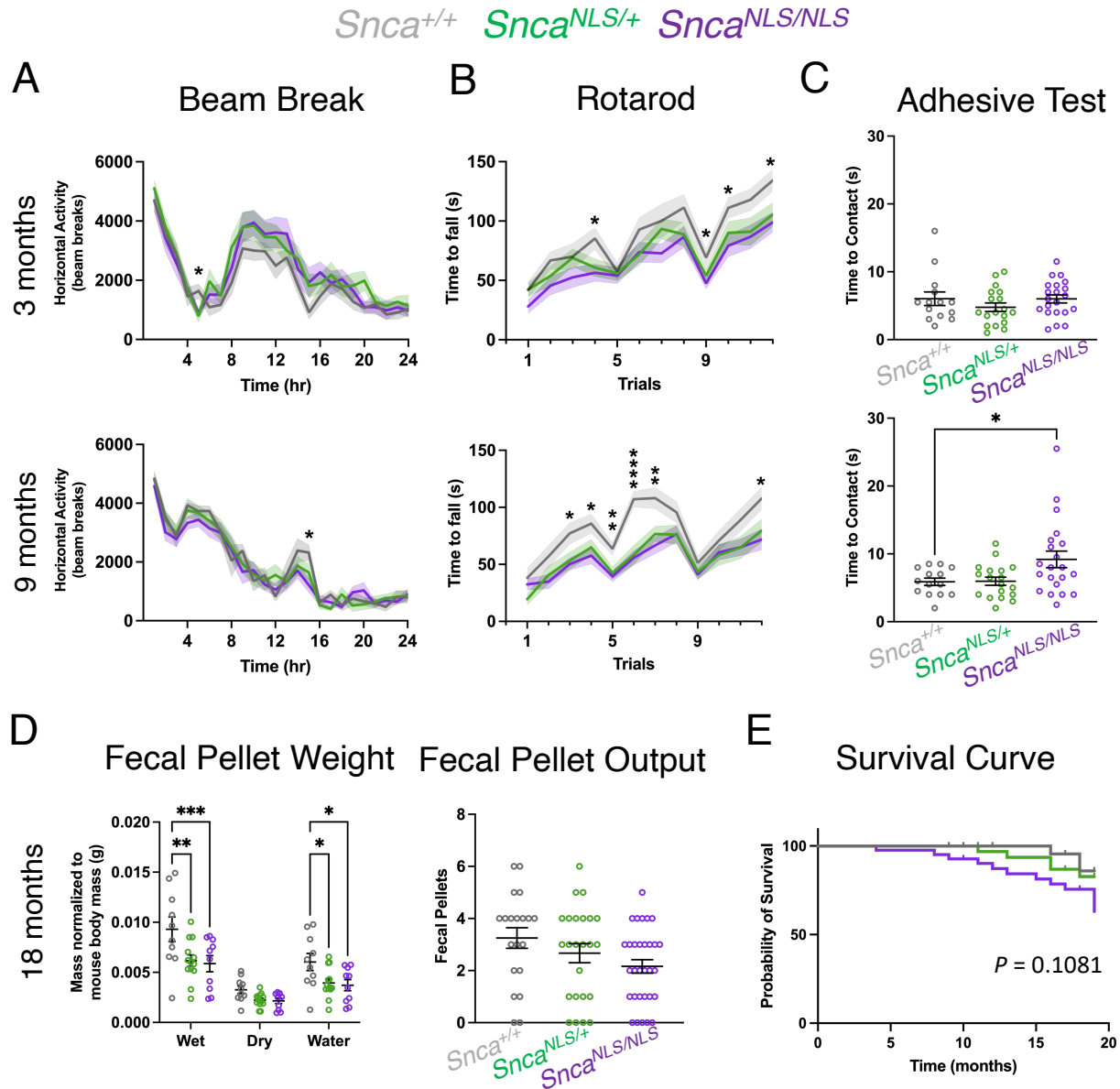
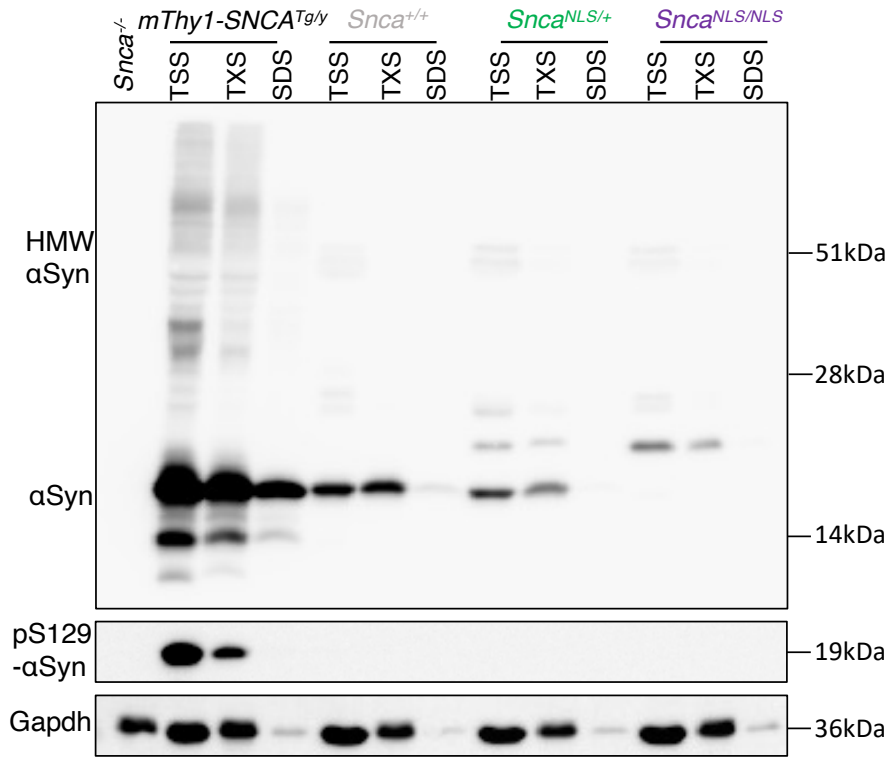


Figure 3: *Snca*^{NLS} mice exhibit significant motor and gastrointestinal dysfunction. Analysis of the 3- (top) and 9-month (bottom) mice for (A) Beam Break, (B) Rotarod and (C) Adhesive Test measuring time to contact their forepaws, (D) Fecal Pellet Weight measuring fecal weight and water content over 1 h (left), and Fecal Pellet Output measuring fecal pellets produced in 10 min (right), ($n = 10-21$). (E) Survival curve from all mice in the behavior colony ($n = 42-58$). One-way (C, D right), two-way ANOVA (A, D left), or mixed-effects analysis (B) with Bonferroni multiple comparison or Log-rank (Mantel Cox) test (F): ns, *, **, *** and **** denote $P > 0.05$, < 0.05 , < 0.01 , < 0.001 and < 0.0001 , respectively.

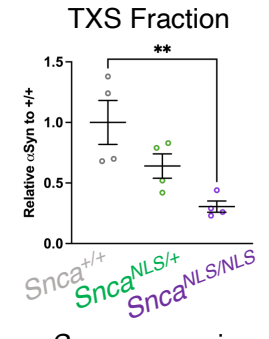
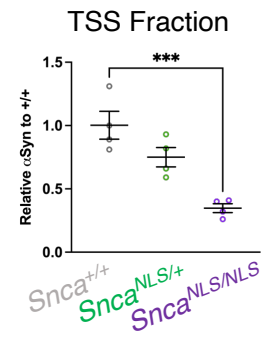
***Snca*^{NLS/NLS} mice exhibit cortical atrophy, independent of α Syn aggregation or dopaminergic neurodegeneration**

Many studies suggest that α Syn toxicity is intrinsically tied to its aggregation, as the two are often associated in humans with PD and in animal models of the disease (Baba et al., 1998; Braak et al., 2003; Spillantini et al., 1997). However, models of α Syn toxicity often rely on the introduction of synthetically derived misfolded α Syn fibrils (Luk, Kehm, Carroll, et al., 2012; Luk, Kehm, Zhang, et al., 2012) or overexpression of α Syn (Chesselet et al., 2012; Masliah et al., 2000), thereby potentiating its aggregation *in vivo*. Given that the *Snca*^{NLS} mice display age-dependent behavioral phenotypes, yet do not rely on α Syn overexpression, we asked whether accumulation of endogenous α Syn in the nucleus leads to its aggregation and thusly contributes to its toxicity. We examined both the solubility of α Syn as well as its pathologically linked phosphorylation at serine residue 129 (pS129) by biochemical fractionation of brain samples of *Snca*^{NLS/NLS} mice compared to littermates. We used the *mThy1-SNCA* (“line 61”) transgenic and *Snca* knockout (*Snca*^{-/-}) mouse lines as positive (Chesselet et al., 2012) and negative (Cabin et al., 2002) controls, respectively. To our surprise, we found that the accumulation of nuclear α Syn does not lead to aggregation (**Fig. 4A**), nor does it become phosphorylated at S129 (**Fig. 4A, B**), and in fact total α Syn levels are reduced in these mice (**Fig. 4A-C**). These findings were further supported by histology, which showed no marked increase in pS129 in aged *Snca*^{NLS/NLS} mice compared to their respective littermates (**Fig. 4D**). This suggests that nuclear accumulation of α Syn confers neuronal dysfunction independent of aggregation.

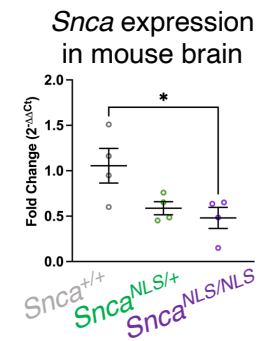
A



B



C



D

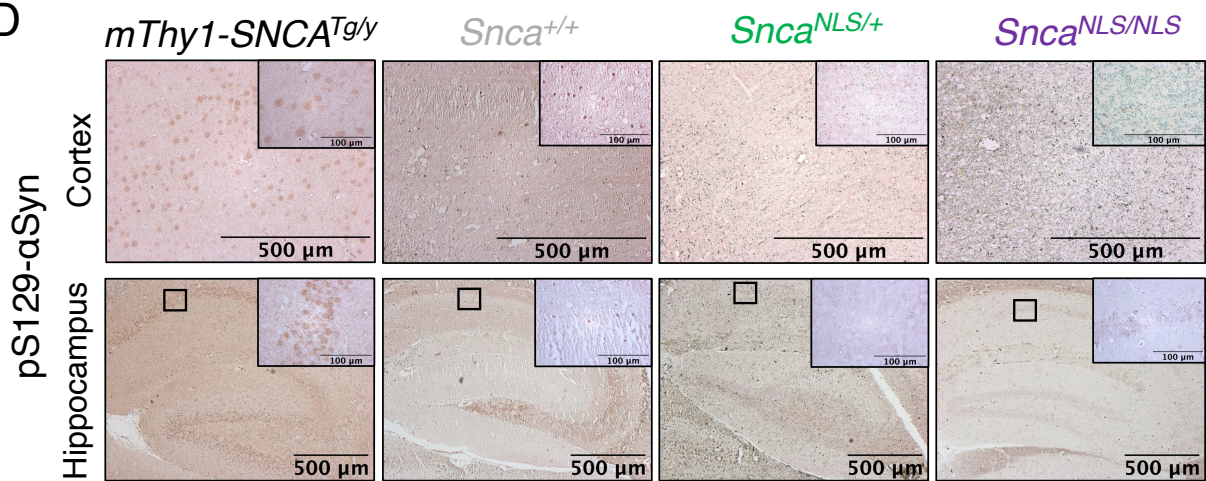


Figure 4: *Snca*^{NLS/NLS} mice do not display significant changes in aggregated or phosphorylated α Syn. Serial extraction of cortical mouse brain tissue with western blot probed for (A) α Syn (upper blot) and pS129- α Syn (middle blot) and Glyceraldehyde-3-Phosphate Dehydrogenase (Gapdh; bottom blot) comparing the level of α Syn in the (B) TSS (upper) and TXS (lower) fraction ($n = 4$). (C) qPCR of *Snca* mRNA from 9-month mouse cortex ($n = 4$). (D) pS129- α Syn staining of the motor cortex (upper) and hippocampus (lower). One-way ANOVA with Bonferroni multiple comparison: *, ** and *** denotes $P < 0.05$, < 0.01 and < 0.001 , respectively.

A hallmark of PD is nigrostriatal degeneration. Due to the relatively high expression of α Syn in dopaminergic neurons (**Fig. 2C**) (Taguchi et al., 2019), we hypothesized nuclear α Syn could be acutely toxic to dopaminergic neurons, causing their death, and ultimately leading to the observed behavioral deficits in *Snc α ^{NLS}* mice. To our surprise, we found that young and aged *Snc α ^{NLS/NLS}* mice had intact nigrostriatal tracts, when evaluated by striatal tyrosine hydroxylase (TH) fiber density and stereological estimation of dopaminergic cell number in the SNc at 3- and 18-months of age (**Fig. 5A-B, Supplementary Figure 4E**). Moreover, HPLC analysis of mouse striata revealed that 18-month-old mice across genotypes exhibit similar levels of dopamine and its metabolites (DOPAC, HVA, 5-HIAA; **Fig. 5C**).

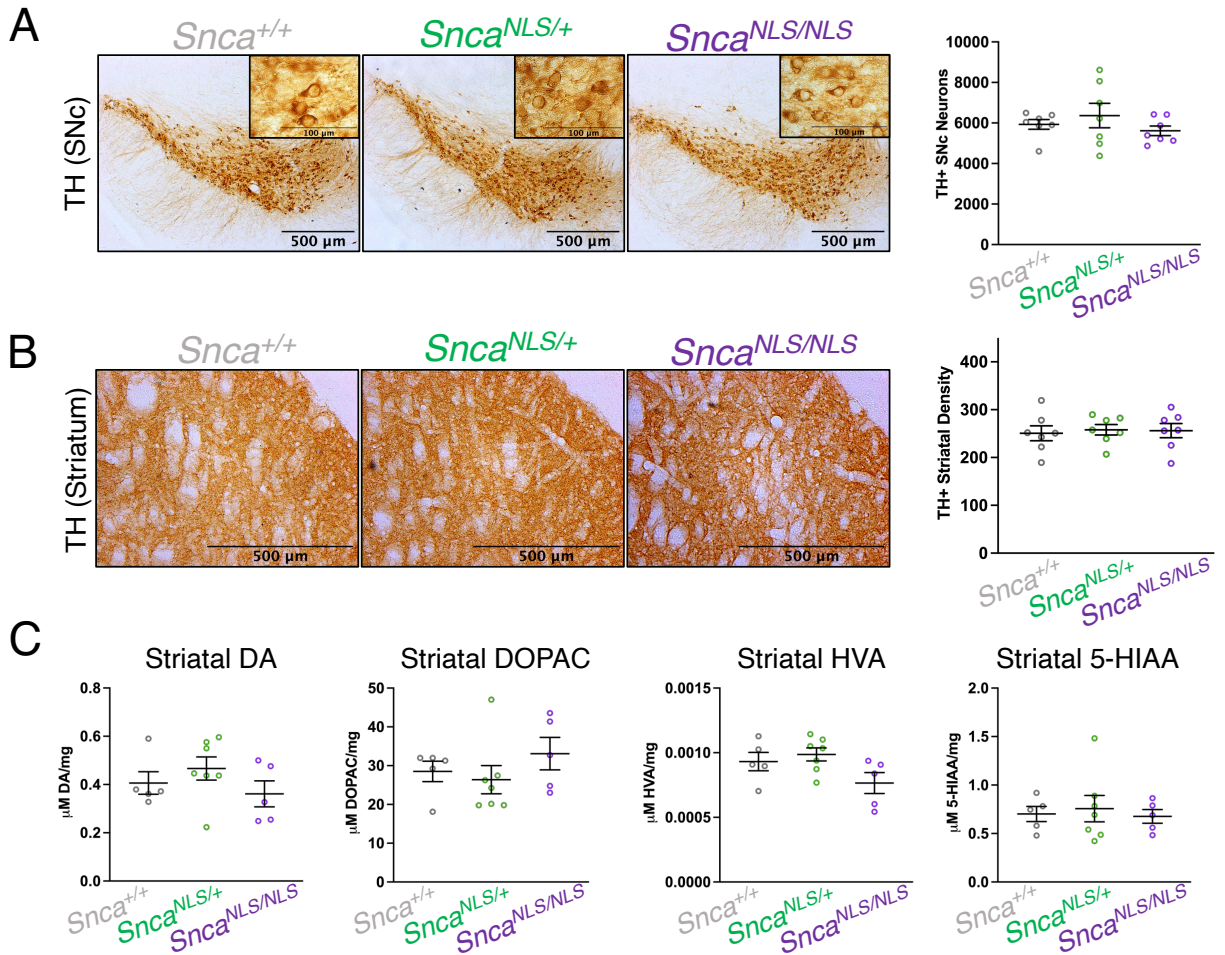


Figure 5: 18-month-old *Snca*^{NLS/NLS} mice exhibit intact nigrostriatal tracts. Tyrosine hydroxylase staining of the (A) Substantia nigra *pars compacta* and (B) striatum of 18-month mice ($n = 7$). (C) HPLC of 18-month striatal tissue measuring Dopamine (left), DOPAC (middle left), HVA (middle right) and 5-HIAA (right) ($n = 5-7$). One-way ANOVA with Bonferroni multiple comparison: ns denotes $P > 0.05$.

Since *Sncα*^{NLS/NLS} mice exhibit motor defects without nigrostriatal degeneration nor αSyn aggregation, we took a step back to ask whether nuclear αSyn may impact other areas of the brain, thus contributing to PD-like phenotypes. Cortical involvement has long been linked to several synucleinopathies including PD, dementia with Lewy bodies (DLB), and PD with dementia (PDD; Braak et al., 2003; Foffani & Obeso, 2018; Giguère et al., 2018; Halliday et al., 2011). We therefore explored higher order cortical areas to determine whether *Sncα*^{NLS/NLS} mice exhibit neurodegenerative features outside of the SNc. We conducted gross anatomical studies using hematoxylin and eosin (H&E) and toluidine blue staining and found significant anterior cortical thinning in the motor cortex (**Fig. 6A**) and a marked increase in pyknotic cells (**Fig. 6B**) throughout the cortex of 18-month-old *Sncα*^{NLS/NLS} mice. We further stained cortical sections with NeuN and found a significant decrease in neurons in layers V/VI of 18-month-old *Sncα*^{NLS/NLS} mice compared to their littermates (**Fig. 6C**). Thus, nuclear αSyn causes cortical dysfunction in the absence of its aggregation or nigrostriatal degeneration.

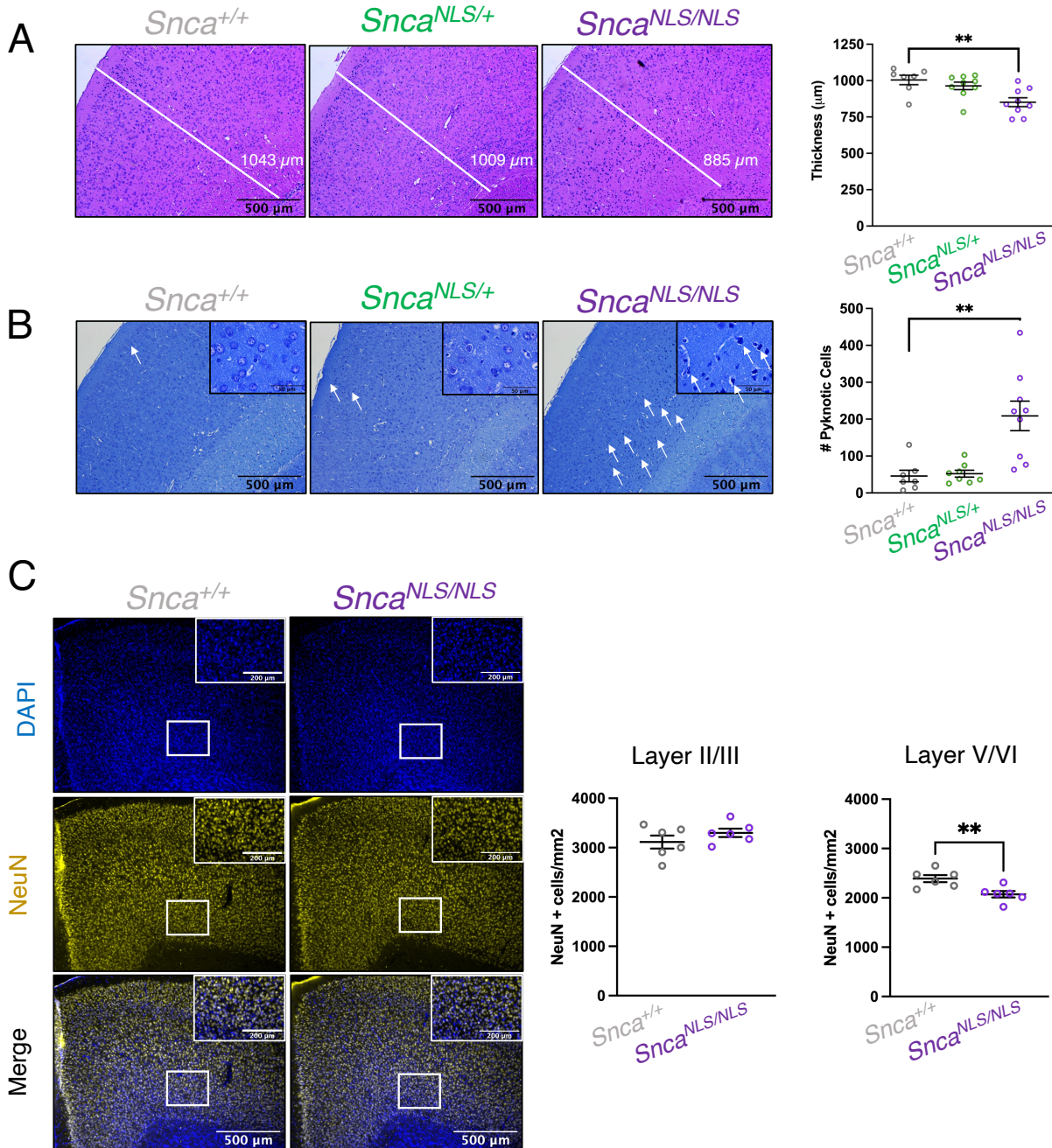


Figure 6: Aged *Snca*^{NLS/NLS} mice exhibit cortical atrophy. (A) H&E staining with quantification of the motor cortex thickness ($n = 7-9$). (B) Toluidine blue staining with quantification of pyknotic cells from the motor cortex ($n = 7-9$). White arrows denote select pyknotic cells. (C) NeuN staining of wildtype (left) and *Snca*^{NLS/NLS} (right) measuring NeuN⁺ cells in motor cortex layers II/III and V/VI. One-way ANOVA with Bonferroni multiple comparison (A, B) or unpaired, two-tailed t -test (C): ns, ** denote $P > 0.05$ and < 0.01 , respectively.

Unbiased proteomic analysis uncovers reduced Darpp-32, Pde10a, and Gng7 levels in *Sncα^{NLS/NLS}* mice

We followed an unbiased strategy to uncover the molecular mechanisms underlying the behavioral and histological phenotypes of the *Sncα^{NLS/NLS}* mice via quantitative proteomic analysis on cortices from 9-month-old mice. At this age, *Sncα^{NLS/NLS}* mice exhibit behavioral abnormalities (**Fig. 3**), allowing identification of early molecular changes that drive the late-stage cortical atrophy exhibited in these mice and in PD. To quantify proteomic differences in wildtype and *Sncα^{NLS/NLS}* mice, we performed pooled TMT10plex labeling for 5 wildtype and 5 *Sncα^{NLS/NLS}* mouse cortices followed by mass spectrometry to identify differential proteomic changes (**Fig. 7A**). This approach yielded a list of nearly 1,800 proteins, of which 114 had a Log₂ fold-change of ± 1 relative to wildtype (**Fig. 7B**). Of these 114 hits, 66 were downregulated and 48 were upregulated (**Supplementary Table 2**). GO term analysis revealed a significant enrichment for biological processes that are disturbed in PD, including regulation of GPCR signalling (**Fig. 7C**). To increase the stringency of our list, we filtered these hits using a statistical cut-off (Mann-Whitney *p*-value < 0.05). From this, we identified 10 high-confidence hits (**Fig. 7D**). Interestingly, among these 10 hits we noticed a few proteins of particular importance in DA signalling and have been associated with PD, such as *Cacna1e*, *Darpp-32*, *Fgf1*, *Gng7*, *Pde10a*, and *Serpina1a* (Benkert et al., 2019; Date et al., 1990; Halbgebauer et al., 2016; Niccolini et al., 2015; Nishi & Shuto, 2017; Schwarzschild et al., 2006; Schwindinger et al., 2010; Torres et al., 2021). We confirmed overall trends in reduction of *Darpp-32*, *Pde10a*, and *Gng7* in the 9-month-old *Sncα^{NLS/NLS}* mice using western blot (**Fig. 7E**), thereby validating our proteomics approach. Collectively, these data suggest that disrupted dopaminergic signalling pathways may be an early event of nuclear α Syn toxicity.

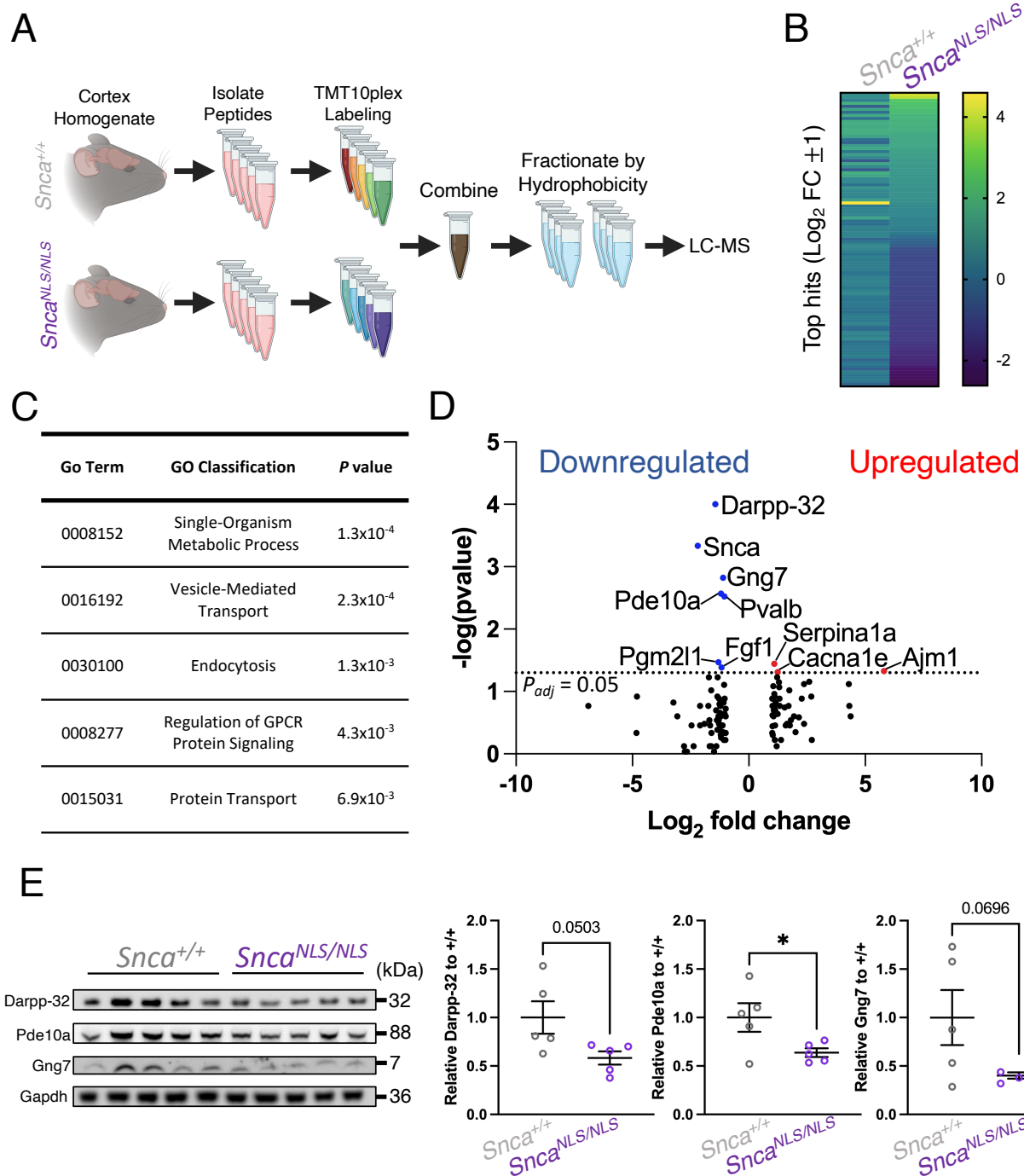


Figure 7: Mass spectrometry reveals proteomic alterations in the *Sncα*^{NLS/NLS} mice. (A) Mass spectrometry scheme for quantitative comparison of proteome between 9-month wildtype and *Sncα*^{NLS/NLS} cortices ($n = 5$). (B) Heat map of all proteins identified through mass spectrometry with values within ± 1 Log₂ fold-change. (C) Table of enriched gene ontology pathways among the ± 1 Log₂ fold-change hits. (D) Volcano plot of mass spectrometry results with ± 1 Log₂ fold-change highlighting significantly upregulated proteins in red and downregulated in blue. (E) Western blot of cortical mouse brain tissue from 9-month mice probing for Darpp-32 (upper), Pde10a (upper middle), Gng7 (lower middle) and Gapdh (bottom) with quantification (right). Unpaired, two-tailed t -test: * denotes $P < 0.05$.

Discussion

The mechanisms underlying α Syn toxicity have been difficult to pin down. We and others have previously shown that nuclear α Syn is increased in the PD post-mortem brain and in animal models harboring *SNCA* mutations (Fares et al., 2014; Jiang et al., 2016; Pinho et al., 2019; Rousseaux et al., 2016; Jiang et al., 2017). Nonetheless, previous studies examining the role of nuclear α Syn in PD pathogenesis have yielded conflicting results, ranging from neurodegenerative (Jiang et al., 2016, 2017; Kontopoulos et al., 2006; Masliah et al., 2000) to neuroprotective (Pinho, Paiva, Jercic, et al., 2019; Schaser et al., 2019) phenotypes. This may be in part due to the degree of overexpression of α Syn or the choice of read-out in these models. Our study sought to overcome this by answering if the nuclear accumulation of *native* α Syn is sufficient to cause PD-like phenotypes in mice. We engineered a mouse model with an *NLS-Flag* knockin on *Snca* to characterize the effects of chronically increased nuclear α Syn. We found that *Snca*^{NLS/NLS} mice reveal PD-like phenotypes including age-dependent motor decline, as well as cortical dysfunction. The cortical atrophy we observed draws parallels to the cell loss seen in synucleinopathies with cortical involvement like DLB and PDD (Braak et al., 2003; Foffani & Obeso, 2018; Giguère et al., 2018; Halliday et al., 2011). Moreover, the anatomical location of this cell loss dovetails with the motor deficits seen in these mice and may shed light onto how nuclear α Syn in PD may be linked to cortical dysfunction and disease manifestation. When examining the proteomic profile of the *Snca*^{NLS/NLS} mice, we found a few high-confidence hits that have been previously associated with PD. Among these, we identified Pde10a, Darpp-32, and Gng7. Although we found no significant decrease in striatal dopamine, it is interesting to note that these proteins are all involved in dopamine signalling, hinting at a potential mechanism by which nuclear α Syn causes dysfunction via perturbing intracellular signaling (Bodén et al.,

2017; Dorsey et al., 2018; Fienberg et al., 1998; Jung et al., 2017; Ma et al., 2021; Niccolini et al., 2015; Nishi et al., 2008; Nishi & Shuto, 2017; Santini et al., 2007; Sasaki et al., 2013; Yger & Girault, 2011). Importantly, *Gng7* knockout mice exhibit significant age-dependent motor deficits, particularly in the rotarod test which was the motor assay with which the *Snca*^{NLS} mice exhibited the most difficulty (Sasaki et al., 2013). Additionally, one study looking at patients with schizophrenia found reduced Pde10a and medial prefrontal cortex thinning, another aspect observed in the *Snca*^{NLS} mice (Bodén et al., 2017). Together, these changes in protein levels hint at a possible mechanism whereby nuclear α Syn elicits dopaminergic signalling defects, in the absence of overt dopaminergic neurodegeneration.

While characterizing *Snca*^{NLS/NLS} mice, we consistently noted how this mouse line diverges from *Snca*^{-/-} mouse phenotypes (Cabin et al., 2002; **Supplementary Figure 2**), cementing that *Snca*^{NLS/NLS} mouse phenotypes are likely gain-of-function. To wit, *Snca*^{-/-} mice exhibit mild synaptic deficits in the absence of gross motor or non-motor deficits (Cabin et al., 2002; Abeliovich et al., 2000), likely due to compensation by β -synuclein and, to a lesser extent, γ -synuclein (Greten-Harrison et al., 2010; Burré et al., 2010; Anwar et al., 2011). Indeed, the motor phenotypes appear to be dependent on the local dose of nuclear α Syn as even the *Snca*^{NLS/+} mice exhibit some motor behavior deficits – albeit to a lesser extent than their *Snca*^{NLS/NLS} littermates, suggesting that they are due to a gain-of-function of nuclear α Syn and not a loss-of-function of synaptic α Syn. Nevertheless, we cannot exclude a model in which partial loss of synaptic α Syn combined with increased nuclear α Syn may drive the age-dependent behavioural and pathological phenotypes seen in *Snca*^{NLS} mice. Strikingly, behavioral and histological phenotypes in *Snca*^{NLS} mice occur independently from α Syn aggregation and pathogenic phosphorylation. Indeed, overall levels of α Syn are reduced in

Snca^{NLS} mice – potentially by some regulatory mechanism or feedback loop – therefore suggesting that these α Syn-mediated phenotypes occur despite its aggregation or phosphorylation. This suggests a heretofore underappreciated role of *soluble*, nuclear α Syn in the pathogenesis of PD.

The cellular mechanisms that drive the nuclear accumulation of α Syn and its subsequent sequelae in PD remain elusive. Whether active or passive mechanisms bring α Syn to the nucleus is unknown. Native α Syn does not possess an NLS, therefore, it may be driven into the nucleus by passive mechanisms (it can traverse the nuclear pore complex due to its small size; Timney et al., 2016) and could be retained by interaction with nuclear components (e.g. histones or DNA; Goers et al., 2003; Kontopoulos et al., 2006; Pinho, Paiva, Jercic, et al., 2019; Paiva et al., 2018; Schaser et al., 2019; Siddiqui et al., 2012) or via uncharacterized modifications. Alternatively, active mechanisms such as its interaction with TRIM28 (Rousseaux et al., 2016) or RAN (Chen et al., 2020) may be key in regulating its nuclear import. Moreover, α Syn likely has an important native role in the nucleus, particularly during mouse embryonic development, where nuclear α Syn constitutes up to 40% of its total cellular distribution, compared to 3-15% of total cellular distribution in adult mice (Pinho, Paiva, Jercic, et al., 2019). There, α Syn is suggested to bind both DNA and histones to modulate gene expression (Goers et al., 2003; Jiang et al., 2018; Kontopoulos et al., 2006; Ma, Song, Yuan, Zhang, Yang, et al., 2014; Paiva et al., 2018; Schaser et al., 2019; Siddiqui et al., 2012; Vasudevaraju et al., 2012). In wildtype mice, nuclear α Syn was shown to be neuroprotective by binding to DNA and colocalizing with DNA damage response elements to protect against DNA damage (Schaser et al., 2019). Whether the increase in nuclear α Syn observed in PD – modeled in the *Snca*^{NLS} mice – causes a gain of this normal

developmental function or a neomorphic function will be important to establish, to facilitate future therapeutic development.

Materials and Methods

Mouse Design and Engineering

Mouse Engineering: To generate the *Snca*^{NLS} mice, Cas9 protein was complexed to a sgRNA targeting the 3' locus of *Snca* (sgRNA target sequence: 5'-TTGGTAGCCTTCCTAATATC-3'); and, together with a single-stranded oligodeoxynucleotide (ssODN) repair template (sequence: 5'- GACAGTTGATATCTGTCACTTCACTGACAAGGCATGCTGTTATTATTTCTTTTTCT GATATTAGGAAGGCTACCAAGACTATGAGCCTGAAGCCCCCCCCAAGAAGAAGAGAA AGGTGGACTACAAGGACGACGACGACAAGTAAGAATGTCATTGCACCCAATCTCCTA AGATCTGCCGGCTGCTCTTCCATGGCGTA -3'; IDT Ultramer), were injected and implanted into pseudo-pregnant FVB female mice (Rousseaux et al., 2018). Three founder mice were generated and backcrossed onto a ~99.75% pure C57Bl6/J background (Taconic 1450 SNP analysis, sequencing and subsequent backcrossing; see **Supplementary Figure 1A, B**), before being expanded onto a mixed C57Bl6/J;C57Bl6/NCrI background. One line was selected for subsequent extensive characterization and is being made available through the Jackson Laboratory (Jax Stock No. 036763).

Genotyping: A small (~1 mm) tail sample is digested prior to PCR amplification using primers outside of the sequence covered by the ssODN used for the initial mouse line. Forward:

5'-TTTTATCTGATTGAAATGATGAGC-3'; Reverse: 5'-ATGACTGGGCACATTGGAA-3'.

PCR protocol: 95 °C for 2 minutes, (95 °C for 30 seconds, 56 °C for 30 seconds, 72 °C for 30 seconds) repeated for 35 cycles, 72 °C for 5 minutes. Mutant allele: 273 bp; Wildtype allele: 225 bp.

Mouse Husbandry

All mice were housed with up to 5 mice per cage on a 12-hour light-dark cycle. Mice were fed *ad libitum*, and all husbandry was performed by the uOttawa Animal Care and Veterinary Services staff. All animal work was done under the approved breeding (CMMb-3009 and CMMb-3654) and behaviour (CMMe-3091) protocols approved under the uOttawa Animal Care Committee. All mice were handled daily for 1 week prior to all behavior testing and both male and female mice were used in all experiments.

Behavior

Open Field: Lighting in the behavior room was set to 100 lux and mice were habituated for 60 minutes prior to testing. Mice were placed into the open field box (45 cm³) for 10 minutes with their movement recorded/analyzed with Ethovision software (Noldus Information Technology). [dx.doi.org/10.17504/protocols.io.b5qzq5x6](https://doi.org/10.17504/protocols.io.b5qzq5x6)

Fecal Pellet Output: Upon completion of the Open Field test, the number of fecal pellets excreted during the 10-minute trial were quantified.

Beam Break: Single mice were placed into a clean cage with access to food and water *ad libitum* for 24 hours at the standard 12-hour light-dark cycle with their movement recorded/analyzed via Fusion software.

Nesting: Directly following Beam Break testing, one square of nestlet (5 cm² cotton pad) was placed in each Beam Break cage for 17-19 hours. Following this, the nestlets were scored on a scale of 1-5 as described in Deacon 2006 (Deacon, 2006), with 1 and 5 representing minimal and maximal nest quality, respectively. [dx.doi.org/10.17504/protocols.io.b5q2q5ye](https://doi.org/10.17504/protocols.io.b5q2q5ye)

Y maze Forced Alternation: Mice were provided with extra-maze (irregular black cue with squared edges on right wall, black triangle on left wall) and intra-maze (Arm 1 has solid black rectangle, Arm 2 has horizontal bars, Arm 3 has diagonal stripes) cues. The room was set to 60 lux and mice were habituated for 60 minutes prior to testing. During the first 5-minute trial, Arms 2 and 3 were alternately blocked. Following the 30-minute inter-trial interval, the mice were placed back into the Y maze apparatus for 5 minutes without any blocked arms and their movements were recorded/analyzed with EthoVision software.

[dx.doi.org/10.17504/protocols.io.b5smq6c6](https://doi.org/10.17504/protocols.io.b5smq6c6)

Adhesive Test: After a 60-minute habituation, the home cage of the mice was lightly wiped to remove all bedding material. The mice were individually placed back into the emptied home cage for a 1-minute habituation. Next, a 1 cm² square of medical adhesive was placed on each forepaw and the mice were placed back into the wiped home cage where the time to remove adhesive was measured up to a 2-minute maximum. [dx.doi.org/10.17504/protocols.io.b5snq6de](https://doi.org/10.17504/protocols.io.b5snq6de)

Pole test: Following a 60-minute habituation, mice were placed on a textured metal pole (8 mm diameter, 55 cm tall) ~3 cm from the top facing upwards. The mice were given up to 1 minute to turn around (facing downwards) and up to 1 minute to descend the pole.

[dx.doi.org/10.17504/protocols.io.b5sqq6dw](https://doi.org/10.17504/protocols.io.b5sqq6dw)

Rotarod: Following a 60-minute habituation, mice were placed on a rod (IITC Life Sciences) rotating from 4-40 revolutions per minute over 5 minutes for 4 trials per day with a 10-minute inter-trial interval. This was repeated for 3 days total. [dx.doi.org/10.17504/protocols.io.b5srq6d6](https://doi.org/10.17504/protocols.io.b5srq6d6)

DigiGait: Following a 60-minute habituation, mice were placed in the DigiGait treadmill (Mouse Specifics Inc.). The treadmill ran at 22 cm/s (3- and 9-months timepoints) or 18 cm/s (18-month timepoint) with a 0° incline. 3 seconds of continuous movement was recorded using DigiGait

Imager software and was then analyzed with DigiGait Analysis software.

[dx.doi.org/10.17504/protocols.io.b5stq6en](https://doi.org/10.17504/protocols.io.b5stq6en)

Fear Conditioning: Naïve age- and sex-matched mice were used to obtain the optimal intensity of foot shock. On day 1 of testing, mice were placed into the Fear Conditioning apparatus (Noldus Information Technology) for 6 minutes during which time the mice experienced 3 tone-shock pairings (30-second tone co-terminated with a 2-second foot shock). On day 2, mice were placed into the same Fear Conditioning apparatus for 6 minutes with no tone or foot shock. On day 3, mice were placed into a different Fear Conditioning apparatus, now altered into a triangular shape with solid floor and a vanilla scent for 3 minutes with no tone then 3 minutes with the same 30-second tone but no foot shock. Freezing was measured/analyzed with EthoVision on all 3 testing days. [dx.doi.org/10.17504/protocols.io.b5suq6ew](https://doi.org/10.17504/protocols.io.b5suq6ew)

Fecal Pellet Composition: Mice were placed in a clean cage and their fecal pellets were collected over a 1-hour period. These pellets were weighed (wet weight) then desiccated at 65 °C for 19 hours and reweighed (dry weight). The differences were calculated between these values to determine the water content. [dx.doi.org/10.17504/protocols.io.b5svq6e6](https://doi.org/10.17504/protocols.io.b5svq6e6)

Histology

See **Supplementary Table 1** for a comprehensive list of antibodies used in this study.

Perfusion: Mice were sedated with 120 mg/kg Euthanyl (DIN00141704) then perfused with 1X phosphate buffered saline (PBS) then 4 % paraformaldehyde (PFA). Brains were then extracted and incubated in 4 % PFA for 72 hours prior to a 3-step sucrose dehydration with 10, 20 and 30 % sucrose (24 hours each). Next, brains were flash frozen for 1 minute in -40 °C isopentane and cryosectioned at 40 µm. [dx.doi.org/10.17504/protocols.io.b5swq6fe](https://doi.org/10.17504/protocols.io.b5swq6fe)

Immunofluorescent staining: Cryosectioned tissue was mounted on a slide then blocked with blocking buffer (0.5% Triton X-100, 10% normal horse serum in 1X PBS) then incubated in primary antibody overnight at 4°C. Next, the sections were incubated in secondary antibody before drying at room temperature for 2 min. The sections were then covered with #1.5 coverslips and fluorescent mounting medium (Dako cat# S3023).

[dx.doi.org/10.17504/protocols.io.b5s5q6g6](https://doi.org/10.17504/protocols.io.b5s5q6g6)

Quick decapitation, fixation: Mice were euthanized by isoflurane inhalation followed by decapitation and brains were quickly extracted. Brains were then submerged in 10 % buffered formalin for 72 hours prior to paraffin embedding and sectioning at 5 µm.

[dx.doi.org/10.17504/protocols.io.b5szq6f6](https://doi.org/10.17504/protocols.io.b5szq6f6)

Diaminobenzidine (DAB) staining: Paraffin-embedded sections were deparaffinized in serial baths of xylenes and ethanol prior to a sodium citrate (10 mM sodium citrate, 0.05 % Tween-20, pH 6) antigen retrieval (20 minutes at 95 °C) and 0.9 % H₂O₂ treatment (10 minutes). Next, sections were blocked in blocking buffer (0.1 % Triton X-100, 10 % normal horse serum in 1X PBS) then incubated in primary antibody overnight at 4 °C. The following day the sections were incubated in secondary and tertiary antibody solution before exposure to DAB, dehydrating in baths of ethanol and xylenes, and covering the tissue with Permount (Fisher Scientific cat# SP15-100) and #1.5 coverslips. [dx.doi.org/10.17504/protocols.io.b5s9q6h6](https://doi.org/10.17504/protocols.io.b5s9q6h6)

Toluidine Blue and H&E staining: Staining was performed by the Louise Pelletier Histology Core facility at the University of Ottawa on paraffin-embedded 5 µm sectioned mouse brain tissue using the Leica Autostainer XL. Briefly, the sections were deparaffinized and exposed to Toluidine Blue for 10 minutes. For H&E, sections were deparaffinized, exposed to hematoxylin for 7 minutes and eosin for 30 seconds. Then, sections were dehydrated and covered with #1.5

coverslips. Two serial sections for each were analyzed by an investigator blinded to the genotypes.

Stereology: Stereology was performed as previously described (Rousseaux et al., 2016b, p. 28). Briefly, for each mouse, 8 cryosections were stained for tyrosine hydroxylase (TH) and quantified using StereoInvestigator software (version 11.06.2). The sections (40 μ m) began at the outer limit of the substantia nigra (SNc) and every 6th section was used (Bregma -2.54 mm to -3.88 mm). Mean section thickness was determined during counting at a frequency of 10 frames (roughly 3 measurements per hemisphere). The SNc was sampled by randomly translating a grid with 150 μ m x 150 μ m squares in the outlined SNc and applying an optical fractionator consisting of a 75 μ m x 75 μ m square. All stereological analyses were performed by an investigator blinded to genotypes. [dx.doi.org/10.17504/protocols.io.b5ynq7ve](https://doi.org/10.17504/protocols.io.b5ynq7ve)

TH Densitometry: For each mouse, 4 cryosections were stained for TH and imaged with Zeiss Axio Imager M2 and analyzed in ImageJ (version 2.3.0/1.53f). The sections (40 μ m) began at the outer limit of the striatum and every 12th section was used (Bregma 1.18 mm to -0.22 mm). Brightfield images were captured at 10X magnification and converted to greyscale. To analyze, an investigator blinded to genotypes averaged 20 intensity measurements within the dorsolateral striatum and normalized to the average of 5 intensity measurements in the corpus callosum (background staining control). All 4 cryosections per mouse were averaged and represent 1 datapoint on the graph. [dx.doi.org/10.17504/protocols.io.b5u4q6yw](https://doi.org/10.17504/protocols.io.b5u4q6yw)

Primary Cortical Neurons: Micro Coverglass #1.5 (Electron Microscopy Sciences) coverslips were pre-coated with poly-D-lysine (50 μ g/mL) for at least overnight at 37 °C, then washed with distilled water twice and air dried at room temperature for two hours. Primary neurons were harvested from E13.5-15.5 pups and seeded at $\sim 1 \times 10^5$ cells per coverslip and cultured for 7 days

in vitro. Cells were fixed in 4% PFA for 20 minutes, washed in 1X PBS, then blocked (10% serum + 0.1% Triton X-100) for 1 hour. Coverslips were stained overnight (see above immunofluorescent staining method). [dx.doi.org/10.17504/protocols.io.b5u6q6ze](https://doi.org/10.17504/protocols.io.b5u6q6ze)

Immunofluorescent imaging: Images for Figures 1 and 2 were obtained on a Zeiss AxioObserverZ1 LSM800 Confocal Microscope. Primary neuron images (Figure 1) were obtained at 40X (oil) magnification at 8bit 1024 x 1024 resolution through a Z distance of ~10 μ m per image using optimal 0.27 μ m spacing. Multichannel acquisition was performed using excitation/emissions: AF488 (Syn1) 493 nm/517 nm (710V); DAPI 353 nm/465 nm (750V); AF647 (Map2) 653nm/668nm (865V); AF594 (α Syn) 280 nm/618 nm (700V). Images from tissue sections (Figure 2) were obtained at 20X magnification (0.8x zoom) at 16bit 1024x1024 resolution through a range of 10 μ m per image using optimal 0.5 μ m spacing. Multichannel acquisition was performed using excitation/emissions (laser intensity); AF488 (α Syn) 493 nm/517 nm (630V for Cortex and DG, 620V for SNc); DAPI 353 nm/465 nm (650V for Cortex and DG, 630V for SNc); AF647 (TH) 653 nm/668 nm (600V). All images were processed and analyzed in ImageJ2 (version 2.3.0/1.53f) using the 3D project and Maximum Z project functions for primary neuron and tissue sections, respectively. Images for Figure 6C were obtained on a Zeiss Axio Imager M2 at 5X magnification and were analyzed in ImageJ2 (version 2.3.0/1.53f).

Nuclear α Syn quantification: Primary cortical neurons were imaged using confocal imaging and all cells in the frame of view were used to quantify the extent of α Syn nuclear localization using ImageJ2 (version 2.3.0/1.53f). First, the nuclei were delineated with DAPI then α Syn intensity was measured within the delineation. This data is shown as the level of α Syn nuclear enrichment relative to wildtype littermates. Cortex, DG, and SNc were analyzed in a similar manner. Briefly,

11-35 cells were sampled per animal using ImageJ2 (version 2.3.0/1.53f), nuclei were defined with DAPI and relative intensity of nuclear α Syn versus cytoplasmic α Syn (immediately adjacent to the nucleus) was measured.

NeuN analysis: Photomicrographs were analyzed using ImageJ2 (version 2.3.0/1.53f). Briefly, NeuN positive structures (neuronal nuclei) were counted in a 200 μ M x 200 μ M frame (Layer II/III) or 350 μ M x 350 μ M frame (Layer V/VI) and then presented as NeuN+ neuron #/mm². Values represent the average from sampling three distinct sections per brain (n=6 per genotype) and were analyzed by an investigator blinded to the genotypes.

Biochemistry

See **Supplementary Table 1** for a comprehensive list of antibodies used in this study.

Serial Extraction: A 0.79 mm³ punch (1 mm thick slice, 1mm i.d. punch) of cortical brain tissue from 18-month-old mice was homogenized and resuspended in a series of increasingly stringent buffers beginning with 100 μ L of TSS Buffer (140 mM NaCl, 5 mM Tris-HCl), then 100 μ L TXS Buffer (140 mM NaCl, 5 mM Tris-HCl, 0.5 % Triton X-100), then 100 μ L SDS Buffer (140 mM NaCl, 5 mM Tris-HCl, 1 % SDS), as previously described (Tokarew et al., 2021). Total protein levels were measured using the PierceTM BCA Assay Kit (Thermo Fisher cat# 23225).

[dx.doi.org/10.17504/protocols.io.b5vvq666](https://doi.org/10.17504/protocols.io.b5vvq666)

Western Blot: Protein samples were loaded into a 12 % polyacrylamide gel and subsequently transferred to a 0.2 μ m nitrocellulose membrane. Membranes were blocked in a 10 % milk solution then incubated in primary antibody (diluted in 2 % bovine serum albumin) overnight at 4 °C. Next, the membrane was incubated in a horseradish peroxidase-conjugated secondary antibody diluted in 10 % milk solution. Then, the membrane was rinsed with ECL Clarity

solution (Bio-Rad cat# 1705061) and imaged with a GE ImageQuant LAS 4000.

[dx.doi.org/10.17504/protocols.io.b5wkq7cw](https://doi.org/10.17504/protocols.io.b5wkq7cw)

RNA Extraction and Real-Time Quantitative PCR: RNA was extracted from mouse brain homogenate using Trizol-Chloroform extraction (Invitrogen™ User Guide: TRIzol Reagent version B.0). Briefly, mouse brains were homogenized in 3 mL of PEPI Buffer (5 mM EDTA, 1X protease inhibitor (GenDEPOT cat# P3100-020), in 1X PBS) using a dounce homogenizer. 3 % of homogenate was added to 1 mL of TRIzol Reagent (Fisher Scientific cat# 15-596-026) and RNA was isolated following the user guide referenced above. cDNA was synthesized using 5X All-in-One RT Master Mix Kit (Bio Basic cat# HRT025-10). Real-time quantitative PCR was performed using Green-2-Go qPCR Master Mix (Bio Basic cat# QPCR004-S) with 25 ng cDNA per reaction and primers targeting mouse *Gapdh* (Forward:

5'-GGAGAGTGTTCCTCGTCCC-3', Reverse: 5'-ATGAAGGGGTCGTTGATGGC-3'),

Hprt1 (Forward: 5'-TGATAGATCCATTCCTATGACTGTAGA-3', Reverse:

5'-AAGACATTCTTCCAGTTAAAGTTGAG-3'), and *Snca* (Forward:

5'-GAAGACAGTGGAGGGAGCTG-3', Reverse: 5'-CAGGCATGTCTTCCAGGATT-3').

Reactions were run on BioRad CFX96 thermocycler (protocol: 95 °C for 5 minutes, 40 cycles of 95 °C for 15 seconds and 60 °C for 60 seconds, then melting curve). *Snca* Ct values were

standardized to the average of *Hprt1* and *Gapdh*. [dx.doi.org/10.17504/protocols.io.b5wmq7c6](https://doi.org/10.17504/protocols.io.b5wmq7c6)

Dopamine and Metabolite Measurement via Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS): Striatal punches (2 mm i.d., 3 mm thick section) were extracted from 18-month old mouse brains and weighed prior to submitting to The Metabolomics Innovation Centre (TMIC). 50 µL of tissue extraction buffer was added to each sample tube followed by homogenization and centrifugation. Supernatant was used for LC-MS/MS analysis to get

concentrations in the unit of μM . TMIC staff applied a targeted quantitative metabolomics approach to analyze the samples using a reverse-phase LC-MS/MS custom assay. This custom assay, in combination with an ABSciex 4000 QTrap (Applied Biosystems/MDS Sciex) mass spectrometer, can be used for the targeted identification and quantification of dopamine (DA), homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5-HIAA), and 3,4-dihydroxyphenylacetic acid (DOPAC). The method combines the derivatization and extraction of analytes, and the selective mass-spectrometric detection using multiple reaction monitoring (MRM) pairs. Isotope-labeled internal standards and other internal standards are used for metabolite quantification. The custom assay contains a 96 deep-well plate with a filter plate attached with sealing tape, and reagents and solvents used to prepare the plate assay. First 14 wells were used for 1 blank, 3 zero samples, 7 standards and 3 quality control samples. For all metabolites except organic acid, samples were thawed on ice and were vortexed and centrifuged at 13,000x g. 10 μL of each sample was loaded onto the center of the filter on the upper 96-well plate and dried in a stream of nitrogen. Subsequently, phenyl-isothiocyanate was added for derivatization. After incubation, the filter spots were dried again using an evaporator. Extraction of the metabolites was then achieved by adding 300 μL of extraction solvent. The extracts were obtained by centrifugation into the lower 96-deep well plate, followed by a dilution step with MS running solvent.

For organic acid analysis, 150 μL of ice-cold methanol and 10 μL of isotope-labeled internal standard mixture was added to 50 μL of sample for overnight protein precipitation. Then it was centrifuged at 13000x g for 20 min. 50 μL of supernatant was loaded into the center of the wells of a 96-deep well plate, followed by the addition of 3-nitrophenylhydrazine (NPH) reagent. After incubation for 2h, BHT stabilizer and water were added before LC-MS injection.

Mass spectrometric analysis was performed on an ABSciex 4000 Qtrap® tandem mass spectrometry instrument (Applied Biosystems/MDS Analytical Technologies, Foster City, CA) equipped with an Agilent 1260 series UHPLC system (Agilent Technologies, Palo Alto, CA). The samples were delivered to the mass spectrometer by a LC method followed by a direct injection (DI) method. Data analysis was done using Analyst 1.6.2.

TMT10plexTM Proteomics via Liquid Chromatography-Mass Spectrometry (LC-MS): Whole mouse cortex was dissected from 9-month-old mouse brain and peptides were isolated using the EasyPepTM Mini MS Sample Prep Kit (ThermoFisher cat# A40006) following manufacturer instructions. These samples were labelled with the TMT10plexTM Isobaric Label Reagent Set (ThermoFisher cat# 90406) then combined into a single tube and fractionated into 12 samples using the PierceTM High pH Reversed-Phase Peptide Fractionation Kit (ThermoFisher cat# 84868). Fractions 2, 3, 9, 10, 11, and 12 were combined due to low protein concentration (combined to have a consistent protein concentration with other fractions) and the 6 final fractions were submitted for LC-MS to the Ottawa Hospital Research Institute Proteomics Core Facility. LC-MS was performed using Orbitrap Fusion Lumos mass spectrometer with UltiMate 3000 RLSC nano HPLC (Thermo Scientific). Proteowizard MS-CONVERT was used to generate peak lists for preliminary qualitative analysis using MASCOT software version 2.7.0 (Matrix Science, UK). Protein identification and quantitative analysis was performed using MaxQuant (Tyanova, Nature Protocols 2016, 11:2301). The reference proteome for peptide spectrum matching was UniProt/Mus musculus (version 2020-10-06). The MaxQuant results were exported to Scaffold Q+S (Proteome Software, USA) for further analysis and viewing. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE ([68](#)) partner repository with the dataset identifier PXD032065

and [{10.6019/PXD032065}](http://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD032065).

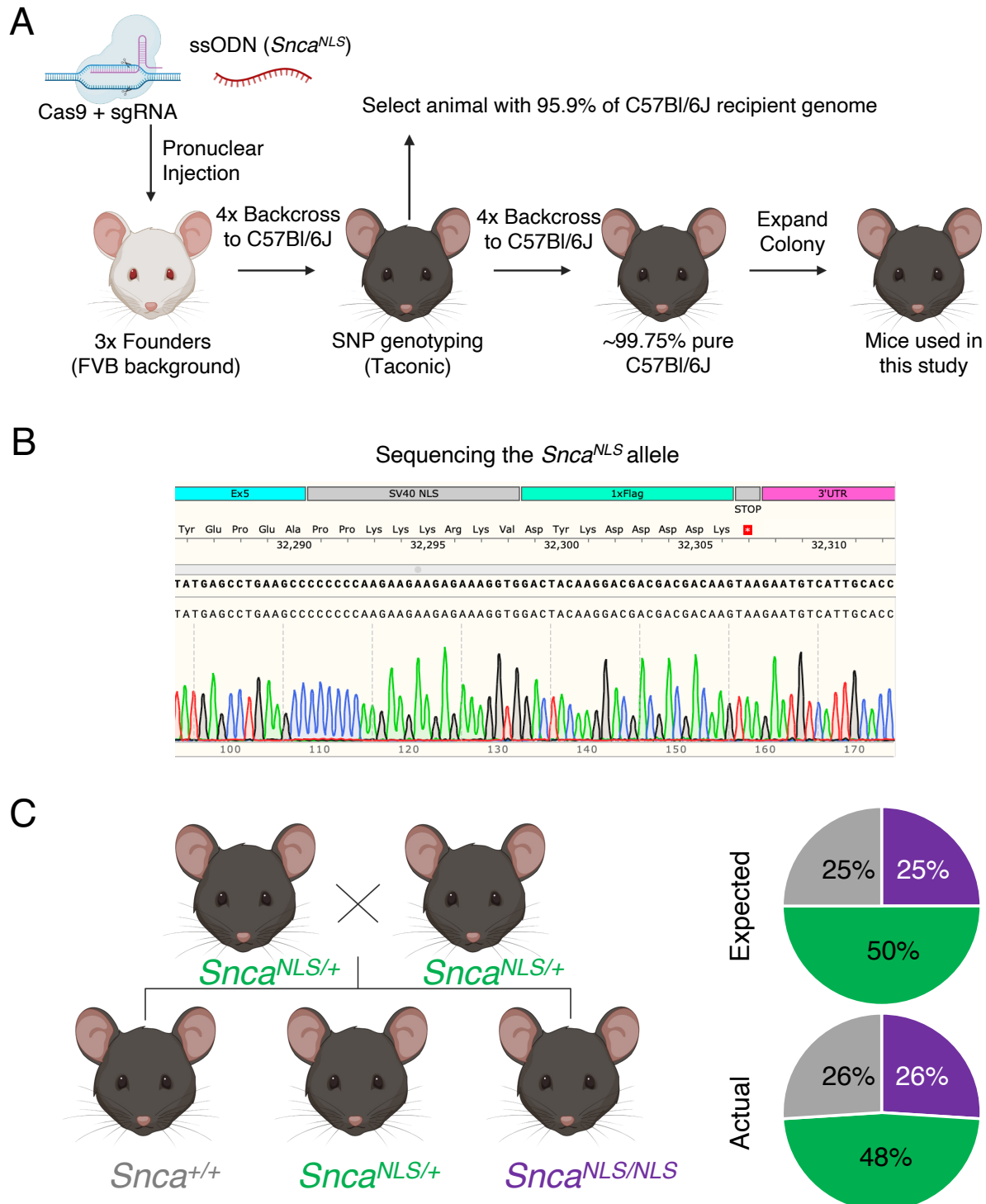
Statistical Analyses

All statistical analyses were performed using GraphPad Prism (version 9.1.2) using the appropriate statistical test, either Student's t-test for simple comparisons or One- or Two-way ANOVA followed by Bonferroni post-hoc analysis for multiple comparisons. The survival curve was analyzed using a log-rank Mantel-Cox test. The Mann-Whitney test with Benjamini-Hochberg correction was used with Scaffold (version 5.0.0) to analyze the TMT10plexTM mass spectrometry dataset. Chi squared test was used to compare actual and expected Mendelian ratios of the genotypes. Statistical tests used, sample sizes, and p-values are delineated in each figure legend. All graphs plot the mean with the standard error of the mean (SEM). All figures and their quantification have been made public ([{doi.org/10.5281/zenodo.6082270}](https://zenodo.org/record/6082270/#.YkuVtNtBzIU)).

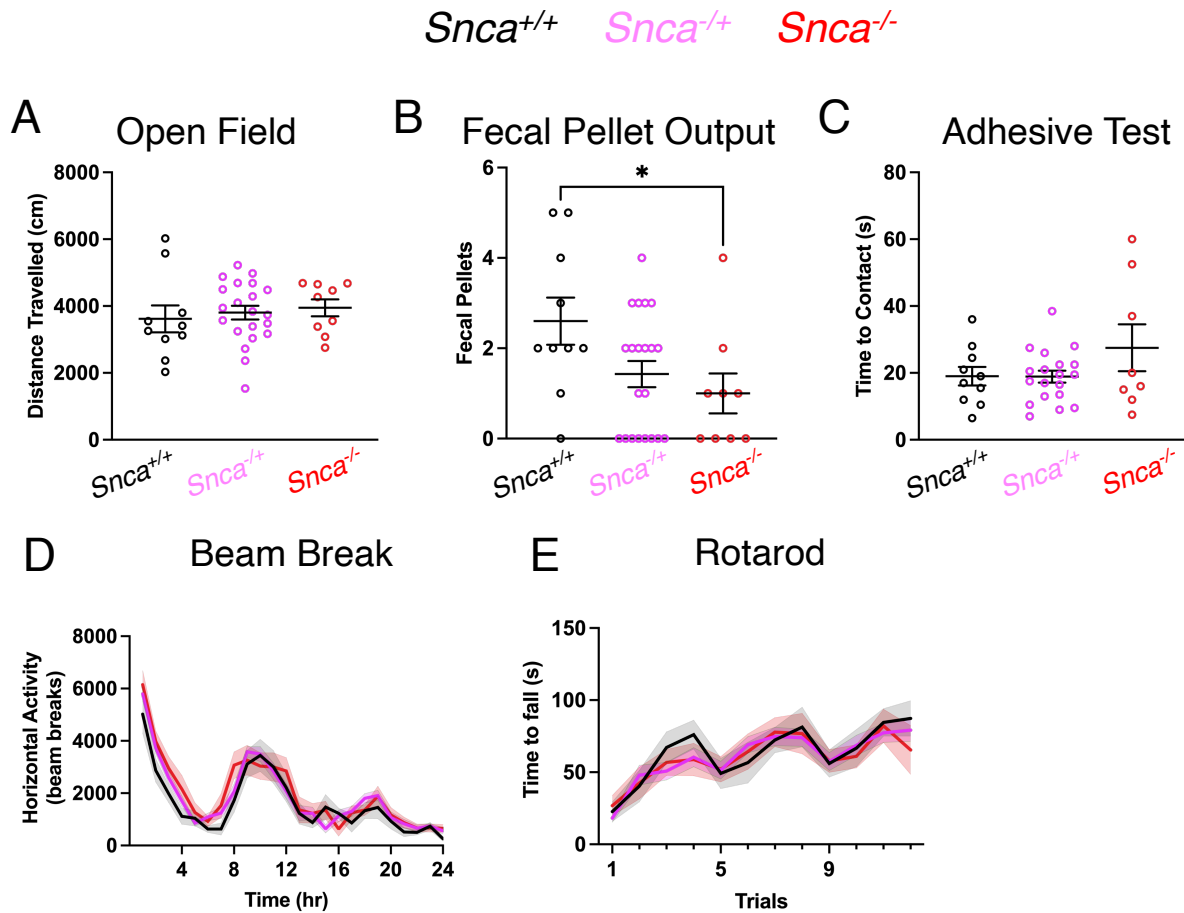
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Supplementary Information

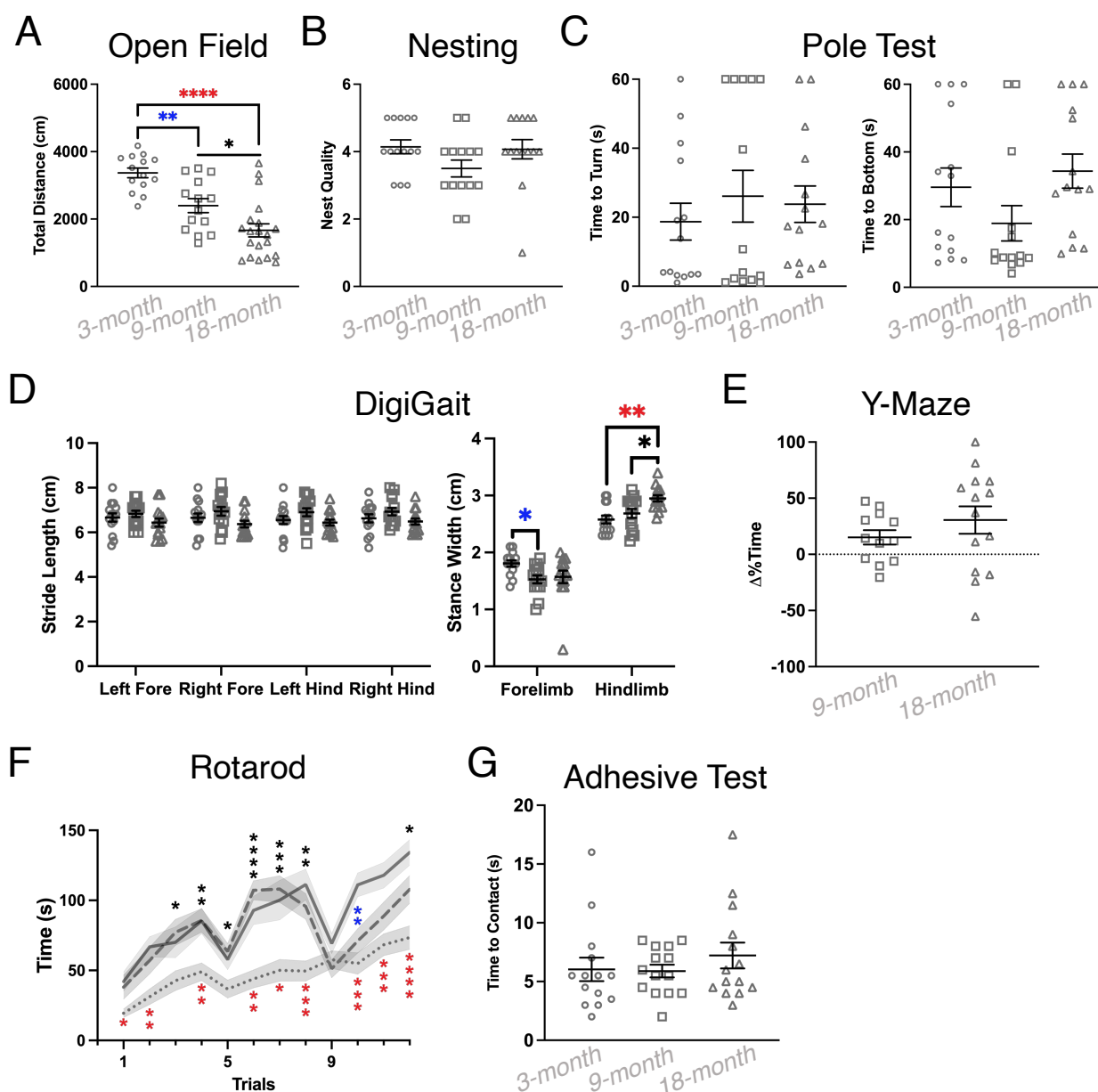


Supplementary Figure 1: Generation of the *Snca^{NLS}* mice. A) *Snca^{NLS}* mouse generation. B) Sequencing confirms the presence of the knock-in. C) Breeding scheme with expected and actual Mendelian ratios among offspring. n = 148 *Snca^{+/+}*, n = 272 *Snca^{NLS/+}*, and n = 147 *Snca^{NLS/NLS}*. Chi squared test: P = 0.7028.



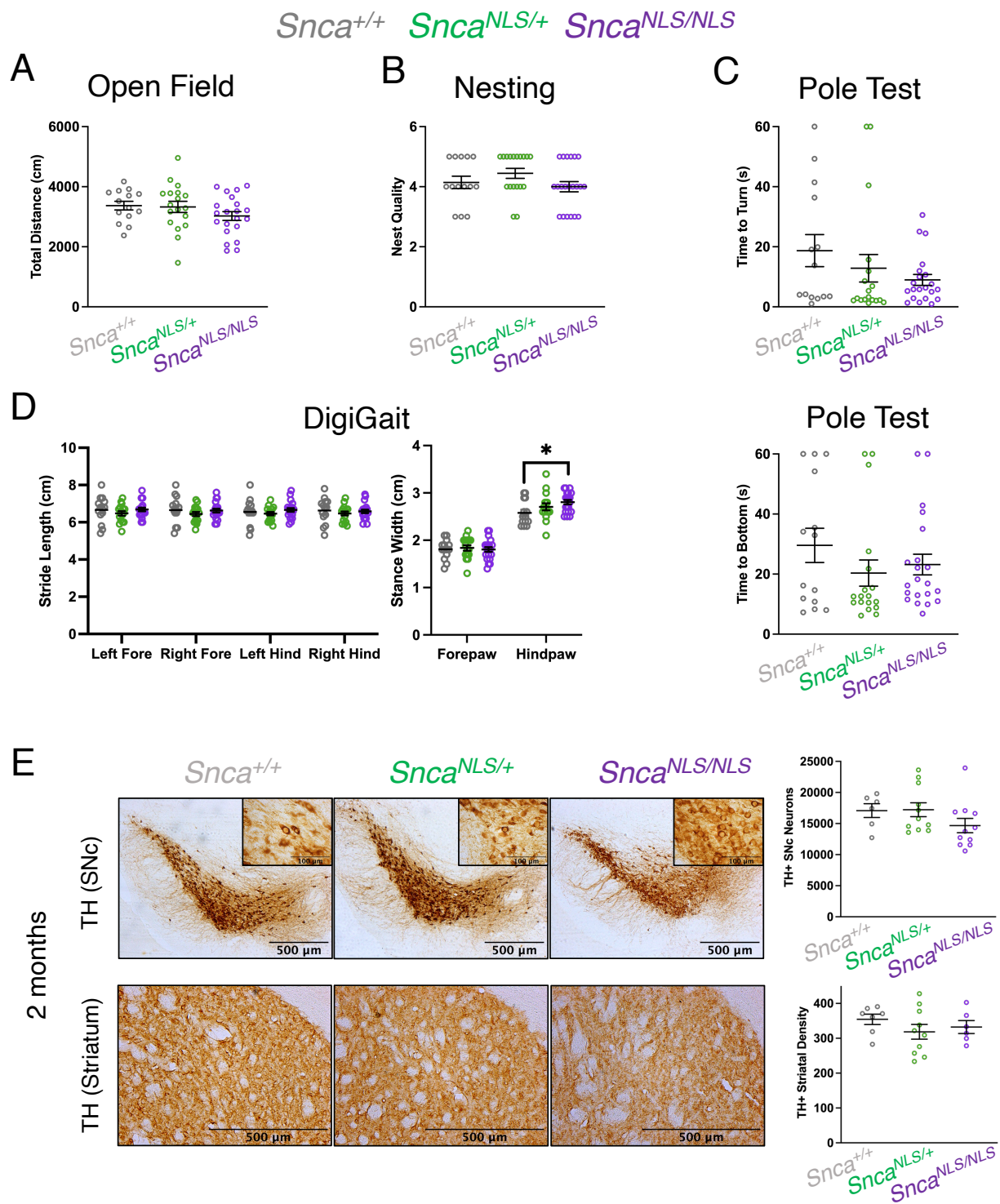
Supplementary Figure 2: Comprehensive behavior profiling of 9-month-old *Snca*^{-/-} mice compared to littermates. Behavior analysis of 9-month *Snca*^{-/-} mice included (A) Open Field, (B) Fecal Pellet Output Test, (C) Adhesive Test, (D) Beam Break measuring horizontal activity, and (E) Rotarod (n=9-21). One- (A,B,C) or Two-Way ANOVA (D,E) with Bonferroni multiple comparison: ns, * denotes $P > 0.05$ and < 0.05 , respectively.

○ 3-Month *Snca*^{+/+} □ 9-Month *Snca*^{+/+} △ 18-Month *Snca*^{+/+}

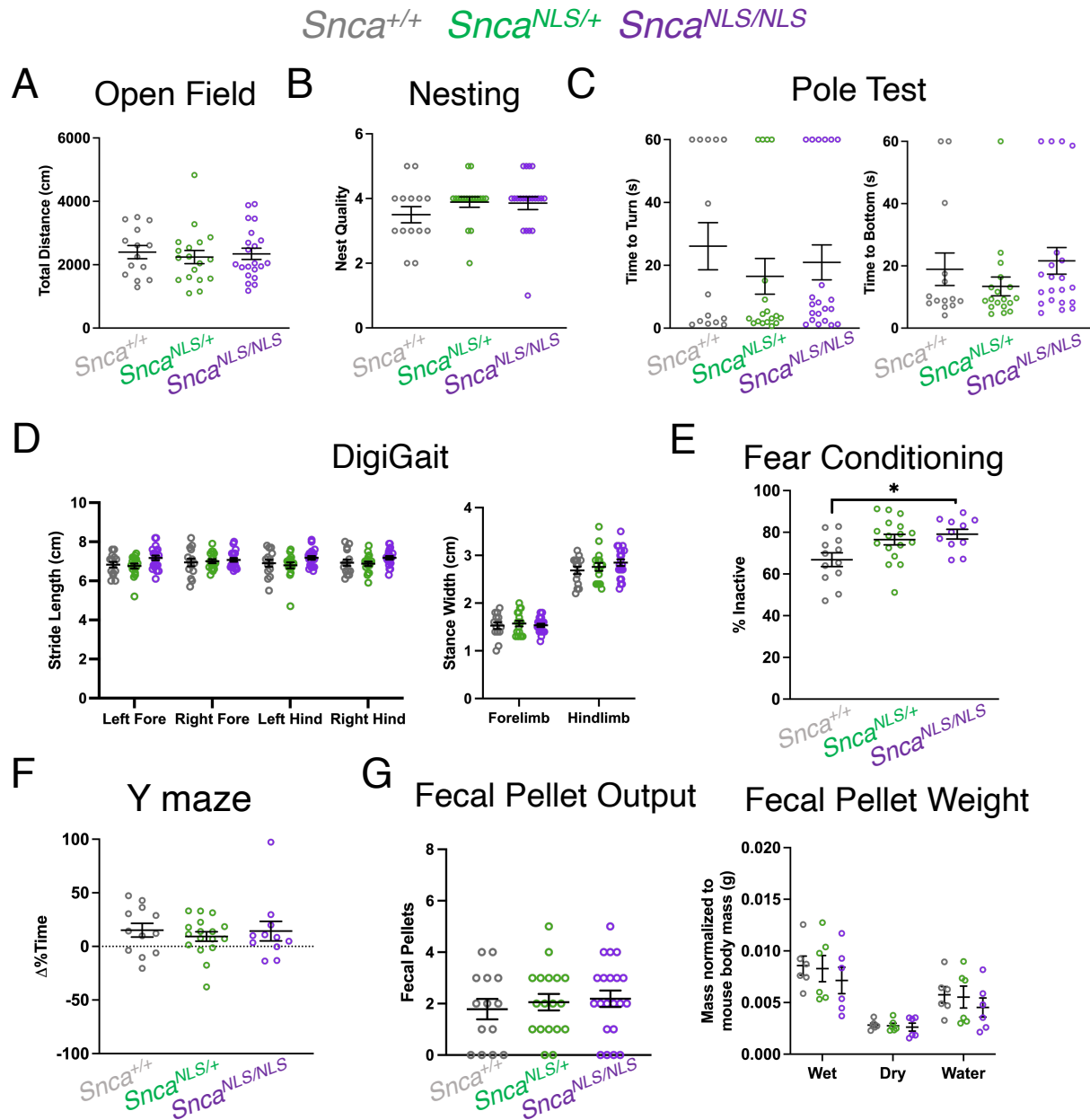


Supplementary Figure 3: Wild-type mice exhibit age-dependent decline in motor ability.

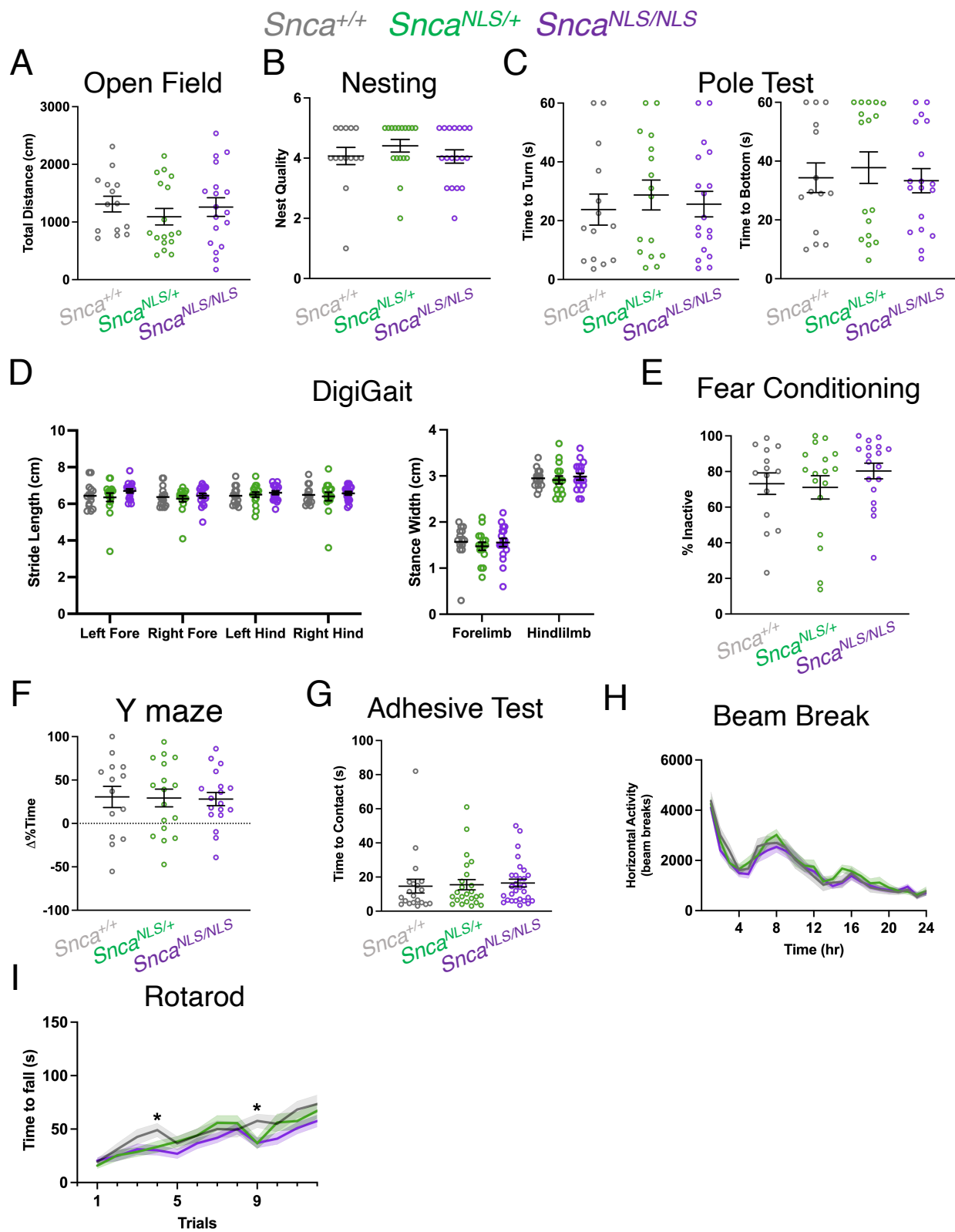
Behavior comparison of wild-type mice at 3-, 9-, and 18-months in (A) Open Field, (B) Nesting, (C) Pole test measuring time to turn over (left) and time to descend to the bottom (right), (D) Digigait measuring stride length (left) and stance width (right), and (E) Y maze (n = 14-20). Motor assays with additional cohorts of mice include (F) Rotarod and (G) Adhesive Test measuring time to contact their forepaws (n = 9-20). One- (A,B,C,E,G) or Two-Way ANOVA (D,F) with Bonferroni multiple comparison: ns, *, **, ***, and **** denote $P > 0.05$, < 0.05 , < 0.01 , < 0.001 , and < 0.0001 , respectively. Blue asterisk denotes significance between 3- and 9-month mice, black asterisk denotes significance between 9- and 18-month mice, and red asterisk denotes significance between 3- and 18-month mice.



Supplementary Figure 4: Comprehensive behavior and dopaminergic profiling of young *Sncα^{NLS/NLS}* mice. Behavior analysis of 3-month mice in (A) Open Field, (B) Nesting, (C) Pole test measuring time to turn over (upper) and time to descend to the bottom (lower), and (D) DigiGait measuring stride length (left) and stance width (right) (n = 14-21). E) Tyrosine hydroxylase staining of the Substantia nigra pars compacta (upper) and striatum (lower) of 2-month mice with their respective quantifications (right) (n = 6-11). One- (A,B,C,E) or Two-Way ANOVA (D) with Bonferroni multiple comparison: ns, * denote $P > 0.05$ and < 0.05 , respectively.



Supplementary Figure 5: Comprehensive behavior profiling of 9-month-old *Snca*^{NLS/NLS} mice compared to littermates. Behavior analysis of 9-month mice in motor assays including (A) Open Field, (B) Nesting, (C) Pole test measuring time to turn over (left) and time to descend to the bottom (right), and (D) DigiGait measuring stride length (left) and stance width (right). Non-motor assays include (E) Fear conditioning, (F) Y maze forced alteration, and (G) Fecal pellet production measuring fecal output in 10 minutes (left) and the weight and water content of fecal pellets produced over 1 hour (right) (n = 14-21). One- (A,B,C,E,F,G left) or Two-Way ANOVA (D,G right) with Bonferroni multiple comparison: ns, * denote P > 0.05 and < 0.05, respectively.



Supplementary Figure 6: Comprehensive behavior profiling of 18-month-old Snca^{NLS/NLS} mice compared to littermates. Behavior analysis of 18-month mice in motor assays including (A) Open Field, (B) Nesting, (C) Pole test measuring time to turn over (left) and time to descend to the bottom (right), and (D) Digigait measuring stride length (left) and stance width (right). Non-motor assays include (E) Fear conditioning and (F) Y maze (n = 14-18). Motor assays with additional cohorts of mice include (G) Adhesive Test measuring time to contact their forepaws, (H) Beam Break measuring horizontal activity, and (I) Rotarod (n = 20-31). One- (A,B,C,E,F,G) or Two-Way ANOVA (D,H,I) with Bonferroni multiple comparison: ns, * denote $P > 0.05$ and < 0.05 , respectively.

Supplementary Table 1: List of antibodies used throughout the study

Antibodies (alphabetical)	Provider, Catalog #, RRID	Species	Application	Concentration	
Primary Antibodies					
α -synuclein	BD Biosciences Cat# 610787, RRID:AB_398108	Mouse	Immunofluorescence	1:2000	
DAPI	Millipore Sigma (D9542)	-	Western blot	1:500 to 1:2000	
Darpp-32	Cell Signaling Technology Cat# 2306, RRID:AB_823479	-	Immunofluorescence	1:1000	
		Rabbit	Western blot	1:1000	
Gapdh	Proteintech Cat# 60004-1-Ig, RRID:AB_2107436	Mouse	Immunofluorescence	1:1000	
Gng7	Thermo Scientific (BS-13246R, RRID:AB_2905542)	Mouse	Western blot	1:100000	
		Rabbit	Western blot	1:500	
hSA4 (α -synuclein)	non-commercial*	Rabbit	Immunofluorescence	1:1000	*non-commercial polyclonal antibody raised and purified against recombinant, full-length human, α -synuclein (as described by Mollenhauer et al, 2008). Of note, its monoclonal antibody is commercially available through Abcam (ab138501)
Map2	Abcam Cat# ab5392, RRID:AB_2138153	Chicken	Immunofluorescence	1:2000	
NeuN	Millipore Cat# MAB377, RRID:AB_2298772	Mouse	Immunofluorescence	1:5000	
Pde10a	Sigma Aldrich (SAB2700582-100UL, RRID:AB_2905543)	Rabbit	Western blot	1:500	
pS129 α -synuclein	Abcam Cat# ab51253, RRID:AB_869973	Rabbit	Immunohistochemistry	1:1000	
			Western blot	1:1000	
Synapsin	Synaptic Systems Cat# 106 001, RRID:AB_887805	Mouse	Immunofluorescence	1:1000	
Tyrosine hydroxylase	Millipore Cat# AB152, RRID:AB_390204	Rabbit	Immunohistochemistry	1:5000	
Secondary Antibodies					
Goat-anti-Chicken IgY (H+L) Alexa Fluor 647	Thermo Fisher Scientific Cat# A-21449, RRID:AB_2535866	Chicken			
Goat-anti-Mouse IgG (H+L) Alexa Fluor 488	Thermo Fisher Scientific Cat# A-11001, RRID:AB_2534069	Mouse	Immunofluorescence	1:500	
Goat-anti-Rabbit IgG (H+L) Alexa Fluor 594	Thermo Fisher Scientific Cat# A-11012, RRID:AB_2534079	Rabbit			
Biotin-SP-AffiniPure Goat-anti-Rabbit IgG (H+L)	Jackson ImmunoResearch Labs Cat# 111-065-003, RRID:AB_2337959	Rabbit	Immunohistochemistry	1:250	
Peroxidase AffiniPure Donkey-anti-Mouse IgG (H+L) Peroxidase	Jackson ImmunoResearch Labs Cat# 715-035-150, RRID:AB_2340770	Mouse			
Peroxidase AffiniPure Donkey-anti-Rabbit IgG (H+L) Peroxidase	Jackson ImmunoResearch Labs Cat# 711-035-152, RRID:AB_10015282	Rabbit	Western blot	1:10000	
Tertiary Antibodies					
Streptavidin-Horseradish Peroxidase Conjugate	Cytiva (RPN1231-2ML)	-	Immunohistochemistry	1:1000	
Staining Kits					
DAB Substrate Kit, Peroxidase HRP, with Nickel (3,3'-diaminobenzidine)	Vector Laboratories Cat# SK-4100, RRID:AB_2336382	-	Immunohistochemistry	-	

Supplementary Table 2: List of hits from proteomic profiling of cortices from 9-month-old *Sncal^{NLS/NLS}* mice compared to wild-type littermates

Identified Protein	Accession Number	Alternate ID	Mann-Whitney U	Log2 Fold Change by Category (NLS-NLS / Reference)
Apical junctional A2AJA9 (+1)	Ajm1		0.047	5.8
Myotubularin G3UYB7 (+3)	Mtmr7		0.25	4.37
Membrane-associated Q80UU9	Pgrmc2		0.17	4.32
Amyloid-beta: B2RUJ5 (+1)	Apba1		0.076	4.29
Adenomatous B2RUG9 (+5)	Apc		0.6	2.71
Cluster of Myl Q3THE2 [2]	Myl12b		0.12	2.68
Glucosaminidase D3YWR1 (+2)	Gnpda2		0.07	2.59
Cytoplasmic O55033	Nck2		0.076	2.37
Protein phosphatase Q8BVQ5	Ppme1		0.33	2.37
Cluster of Integrin A0A2R8VK7C	Cd47		0.13	2.27
Eukaryotic translation Q9WUK2 (+1)	Eif4h		0.26	2.15
Cluster of Tyrosine Q8CCT4 [4]	Tceal5		0.45	2.01
Tyrosine-protein P35235 (+1)	Ptpn11		0.25	1.94
Monofunctional Q3V3R1	Mthfd1l		0.29	1.8
Lamina-associated Q61029 (+5)	Tmpo		0.35	1.76
Protein FAM108 Q80VD1	Fam98b		0.096	1.74
Interleukin epsilon Q9CXY6	Ilf2		0.17	1.71
Tubulin-specific Q8BYA0	Tbcd		0.33	1.64
BTB/POZ domain A0A1D5RL96	Btbd8		0.35	1.58
Integrin alpha A2AKI5 (+1)	Itgav		0.18	1.54
Glutathione S-transferase A2AQN2 (+1)	Ggt7		0.6	1.38
Importin subunit O35344	Kpna3		0.17	1.33
Cluster of Calcium F8WHB1 [4]	Atp2b2		0.13	1.32
Secretory carrier E9Q855 (+2)	Scamp3		0.085	1.3
Vacuolar protein Q3UVL4 (+1)	Vps51		0.071	1.29
Voltage-dependent A0A087WS8	Cacna1e		0.049	1.24
Heterogeneous Q99020	Hnrnpab		0.23	1.24
Eukaryotic protein Q8BWW3	Etf1		0.059	1.22
Calmodulin-related A0A0A6YY67	Camsap2		0.75	1.19
GTP-binding Q921J2	Rheb		0.15	1.15
ADP-ribosylase V9GWV1 (+1)	Arfgap1		0.2	1.13
Cullin-2 OS=I Q9D4H8	Cul2		0.2	1.13
Actin-binding E9Q0W6 (+1)	Ablim2		0.35	1.13
Vesicular glutamate Q3TXX4	Slc17a7		0.2	1.12
Coiled-coil domain Q3UHB8	Ccdc177		0.6	1.12
ELAV-like protein A0A1L1SS95	Elavl3		0.12	1.11
Alpha-1-antitrypsin A0A0A0MQA	Serpina1a		0.036	1.09
Phosphoribosyl Q8R574	Prpsap2		0.11	1.09
Hepatoma-derived Q9JMG7 (+1)	Hdgfl3		0.17	1.09
Cluster of Heat O35737 [4]	Hnrnp1		0.2	1.07
Signal transducer P70297 (+1)	Stam		0.25	1.06
Metaxin OS=D3YTP3	Mtx3		0.082	1.05
ADP-ribosylase A2A9W7 (+2)	Gga3		0.5	1.03
Ataxin-2-like A0A0U1RPLC	Atxn2l		0.17	1.02
Leucine-rich A0A0G2JDT9	Lrrc7		0.24	1.02
ATP-dependent J3QMM7 (+4)	Naxd		0.36	1.02
Bifunctional Q8CGC7	Eprs1		0.13	1
Protein Hook Q8BUK6	Hook3		0.46	1
Cluster of Myl Q8CAQ8 [4]	Immt		0.19	-1
Hepatocyte gamma B1ATY9	Hgs		0.25	-1
Lactoylglutamate Q9CPU0	Glo1		0.25	-1
Arf-GAP with E9Q3K3	Agap1		0.46	-1
Potassium voltage P63141	Kcna2		0.46	-1.01
Clathrin intermediate Q55UH6 (+3)	Clint1		0.6	-1.01
Programmed P12815 (+1)	Pdcd6		0.13	-1.04
Retinol-binding Q00915	Rbp1		0.23	-1.04
Parvalbumin P32848	Pvalb		0.003	-1.05

10 high-confidence hits

Isoform 3 of Q9ES97-3	Rtn3	0.46	-1.05
Protein-tyros B9EJA3 (+16)	Ptprd	0.078	-1.06
Leucine-rich E9PV22 (+1)	Lrrc47	0.6	-1.07
Xaa-Pro dipe Q11136	Pepd	0.15	-1.08
Guanine nucl G3X9Q2 (+1)	Gng7	0.0015	-1.11
Y-box-binding A2BGG7 (+1)	Ybx1	0.45	-1.11
Coiled-coil dk E0CYP1 (+3)	Ccdc127	0.35	-1.13
Wiskott-Aldr Q8VHI6	Wasf3	0.45	-1.13
Fibroblast gr P61148	Fgf1	0.041	-1.17
Phosphodies A0A384DV92	Pde10a	0.0027	-1.19
Ceramide tra Q9EQG9 (+2)	Cert1	0.35	-1.19
UMP-CMP ki Q3U5Q7	Cmpk2	0.36	-1.19
AW555464 p B9EIX2 (+1)	Cep170b	0.55	-1.2
Keratin, type Q3UV17	Krt76	0.25	-1.21
BTB/POZ dor A0A0R4J2B2	Kctd12	0.5	-1.21
Purine nucle P23492 (+1)	Pnp	0.17	-1.22
Voltage-dep E9Q683 (+6)	Cacna2d2	0.17	-1.23
Contactin-2 C Q61330	Cntn2	0.22	-1.23
Target of My Q88746 (+1)	Tom1	0.16	-1.26
Ras-related j P61021	Rab5b	0.25	-1.26
Serine/argini A0A3Q4EH0	Srsf7	0.13	-1.28
Receptor-ty B0V2N1 (+4)	Ptprs	0.33	-1.28
Leucine--tRN Q8BMJ2	Lars1	0.29	-1.29
Glucose 1,6- Q8CAA7	Pgm2l1	0.034	-1.3
TFG protein i Q9Z1A1	Tfg	0.41	-1.3
Protein FAM: Q8BR63	Fam177a1	0.25	-1.31
Constitutive i F8WGF2 (+6)	Nos1	0.41	-1.31
Tripartite mc Q3TDT0 (+1)	Trim3	0.059	-1.34
Epidermal gr Q60902 (+2)	Eps15l1	0.12	-1.35
WASH compl Q3UMB9	Washc4	0.41	-1.35
Aldehyde del Q80VQ0	Aldh3b1	0.29	-1.37
Chromodom A2A8L1 (+2)	Chd5	0.75	-1.39
Omega-amic A0A338P6G	Nit2	0.2	-1.41
Protein phos Q60829 (+1)	Ppp1r1b	< 0.0001	-1.44
Microtubule- A0A1D5RLY6	Map1s	0.92	-1.5
VPS10 doma Q9EPR5	Sorcs2	0.46	-1.61
Mitochondri Q8BG51 (+3)	Rhot1	0.096	-1.62
Kinectin OS= A0A087WNV	Ktn1	0.75	-1.62
1-acyl-sn-gly A0A0R4J263	Agpat1	0.35	-1.66
Kinesin-like j B1AQZ2 (+3)	Kif3a	0.75	-1.68
WAS/WASL- D3Z5R4 (+3)	Wipf3	0.29	-1.69
Anillin OS=M Q8K298	Anln	0.059	-1.72
Tuberin OS=I A0A2I3BPP1	Tsc2	0.17	-1.74
Mitochondri Q9CXT8	Pmpcb	0.33	-1.9
Protein lin-7 Q88952	Lin7c	0.35	-2.09
Glucosidase Q08795 (+1)	Prkcsb	0.17	-2.1
Alpha-synucl Q55042 (+1)	Snca	0.00046	-2.19
Guanylate cy F8VQK3	Gucy1a2	0.75	-2.37
Hydroxystero B1AX78 (+1)	Hsd12	0.35	-2.46
N(G),N(G)-di Q99LD8	Ddah2	0.92	-2.66
Vacuolar pro A0A2R8VKSE	Vps28	0.92	-2.75
Sodium chan A0A0J9YUW	Scn8a	0.75	-2.78
Coiled-coil dk D3YZP9	Ccdc6	0.25	-3.07
Phosphoribo B1AT82 (+1)	Prpsap1	0.15	-3.24
Sorting nexin Q70493 (+3)	Snx12	0.12	-4.81
Phosphatidyl A0A5F8MPK	Sacm1l	0.46	-4.83
Delta-aminol P10518	Alad	0.17	-6.89

Chapter 3: Nuclear alpha-synuclein interactome reveals a biochemical and functional interaction with the RNA binding protein Luc7l

Manuscript in preparation

Geertsma HM, Brain DM, Nguyen B, Callaghan SM, Caballero A, Rousseaux MWC

Statement of author contribution

This manuscript catalogs the nuclear-specific interactome of α Syn, as compared with its wild-type and cytosol-specific interactome. We found that the nuclear α Syn interactome is enriched for proteins involved in the spliceosome. From this, we validated Luc7l, an RNA-binding protein of the U1 snRNP splicing complex, using bimolecular fluorescent complementation and co-immunoprecipitation assays. Furthermore, we noted that *LUC7L* overexpression in cell models led to an overexpression of α Syn, suggesting LUC7L can stabilize α Syn protein. We also explored the expression and colocalization of α Syn and Luc7l in both the *Snc α ^{NLS}* mice and PD post-mortem brain tissue. Here, we found that Luc7l colocalizes with nuclear α Syn in the mice, and that LUC7L intensity is greater in PD brain slices relative to non-neurodegenerative controls. This suggests a possible mechanism by which LUC7L stabilizes α Syn in PD, contributing to α Syn accumulation and aggregation.

Haley Geertsma and Dr. Maxime Rousseaux conceptualized the study. **Haley Geertsma**, Devin Brain, and Steve Callaghan generated all plasmids used in this study. **Haley Geertsma** and Ben Nguyen cultured all primary cortical neurons used in this study. **Haley Geertsma** performed and analyzed all TurboID experiments. **Haley Geertsma** performed all immunofluorescent experiments, including imaging and analysis. Ben Nguyen performed all flow cytometry experiments with the help of Alex Caballero. **Haley Geertsma** and Devin Brain performed all western blot and immunoprecipitation assays. **Haley Geertsma** and Dr. Maxime Rousseaux wrote the manuscript and all authors provided edits.

Nuclear alpha-synuclein interactome reveals a biochemical and functional interaction with the RNA binding protein Luc7l

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Abstract

Alpha-synuclein (α Syn) abnormally accumulates in diseases of the brain, collectively termed α -synucleinopathies. While a clear relationship between α Syn accumulation and disease exists, it remains unclear how this accumulation is linked to neurodegeneration. We and others have described that nuclear α Syn accumulation is one potential mechanism that drives disease pathogenesis. To test how α Syn nuclear localization and accumulation may be tied to disease, we performed TurboID proximity labelling coupled with mass spectrometry to uncover novel α Syn nuclear interactors. We discovered that the nuclear α Syn interactome was enriched for several spliceosome and RNA-binding proteins, including Luc7l. We used orthogonal approaches to validate this interaction, including co-immunoprecipitation, bimolecular fluorescent complementation (BiFC) and co-immunofluorescent staining of tissue. In each case, we found a tight association between α Syn and Luc7l. To our surprise, Luc7l overexpression alone was found to increase the steady state levels of α Syn and that LUC7L may be elevated in PD post-mortem brain tissue. Altogether, the interaction between α Syn and LUC7L may be important for nuclear α Syn function and provides an untapped avenue for exploring this interaction in disease.

Introduction

Alpha-synuclein (α Syn) is a central protein involved in Parkinson's disease (PD), among other synucleinopathies. In PD, α Syn is a hallmark of disease via its accumulation in the form of intracellular, cytoplasmic aggregates termed Lewy bodies and Lewy neurites (Spillantini et al., 1997b). Although it was first described as a presynaptic and nuclear protein, its role in the nucleus, both natively and its contribution to disease, have been largely understudied (Maroteaux et al., 1988a). In healthy states, there is evidence to support a neuroprotective role, with α Syn aiding in double stranded DNA break repair (Schaser et al., 2019a; Arnold et al., 2024b). However, it is thought that the abnormal accumulation of nuclear α Syn may also contribute to disease (McLean et al., 2000; Specht et al., 2005; Kontopoulos et al., 2006). Previous studies have shown nuclear accumulation of α Syn in post-mortem disease brains, and animal models that replicate this nuclear accumulation exhibit neurotoxicity (Geertsma et al., 2022; Kontopoulos et al., 2006; Koss et al., 2022; Pan et al., 2022; Rousseaux et al., 2016). However, the mechanism(s) of this toxicity remain unknown. Recently, our lab generated and characterized a mouse model (*Snc^{aNLS}*) that mislocalizes endogenous α Syn to the nucleus and found significant motor deficits and cortical neurodegeneration without toxic α Syn aggregation nor phosphorylation at Serine-129, suggesting a toxic gain-of-function of nuclear α Syn (Geertsma et al., 2022). We hypothesize that α Syn forms new interactions or disrupts existing protein-protein interactions upon its nuclear accumulation, thereby leading to neurotoxicity. Understanding the nuclear α Syn interactome can provide meaningful insight into the potential mechanisms for its nuclear-specific role in healthy and disease states. Previous α Syn interactomes have largely focused on its cytoplasmic localization, or its interactome following different post-translational modifications (Chung et al., 2017; Griffin et al., 2023). However, there remains a gap in

knowledge in how the subcellular milieu may define α Syn interactions. To better ascertain the nuclear-specific α Syn interactome, we performed α Syn-TurboID proximity labelling coupled with immunoprecipitation and mass spectrometry to identify proteins that interact with α Syn within the nucleus. In parallel, we also performed Flag immunoprecipitation (IP) coupled with mass spectrometry from *Snca*^{Flag}, *Snca*^{NLS}, and *Snca*^{NES} mouse primary cortical neurons. These mice have a knock-in of a Flag, NLS-Flag, or NES-Flag tag on the 3' end of the *Snca* coding sequence, respectively. In both TurboID and Flag IP datasets, we find significant enrichment of proteins involved in RNA binding and the spliceosome pathway. Notably, we identified Luc7l, an RNA-binding protein of the U1 snRNP splicing complex, as a novel α Syn interactor. We found that it forms a complex with α Syn *in vitro* and *in vivo*. Interestingly, we also find that Luc7l overexpression leads to an overabundance of α Syn protein.

Results

TurboID proximity labelling can identify protein interactors with α Syn in different cell compartments

Previous studies have found that the nuclear accumulation of α Syn is neurotoxic and can contribute to disease. However, the precise role of nuclear α Syn, and its role in disease, remain unknown. We hypothesize that the nuclear-specific protein-protein interactions could be contributing to disease. To study its role in the nucleus, α Syn is typically overexpressed with a nuclear localization tag to induce nuclear mislocalization, but this can lead to confounding effects due to its propensity for aggregation when overexpressed and the presence of an untagged, competing native protein. To overcome these issues, we overexpressed *SNCA-NLS* and other variants in *Snca* knockout (*Snca*^{KO}) neurons at a low multiplicity of infection (MOI) to complement for the loss of gene, essentially humanizing these neurons for different TurboID construct. This helps avoid competing with endogenous α Syn and to express *SNCA* at physiologically-relevant levels to reduce the likelihood of aggregation.

To identify novel nuclear α Syn interactors, we used TurboID, a biotin ligase that uses ATP to convert biotin into biotin-AMP to covalently label proteins within a small ~40 nm radius (**Fig 1a**; Cho et al., 2020). We generated three lentiviral plasmids to acquire the interactomes within different subcellular distributions of α Syn and used a V5 tag for downstream applications: *SNCA-V5-TurboID* (wild-type distribution), *SNCA-NLS-V5-TurboID* (nuclear localized), and *SNCA-NES-V5-TurboID* (nuclear excluded; **Fig 1b**). We also generated three lentiviral plasmid controls to account for non-specific binding of proximal proteins: *GFP-TurboID*, *GFP-NLS-TurboID*, and *GFP-NES-TurboID* (**Fig 1b**). We generated lentiviruses of these vectors and transduced *Snca*^{KO} primary cortical neurons upon plating in 6-well dishes. Neurons were

transduced with viruses for 4 days before fully changing the media to biotin-free media. On day 7, we added 250 μ M biotin for 10 minutes then isolated all biotinylated proteins for mass spectrometry analysis (proof of concept is presented in **Fig 1c**). This was sufficient to induce significant biotinylation for subsequent immunoprecipitation and mass spectrometry analysis.

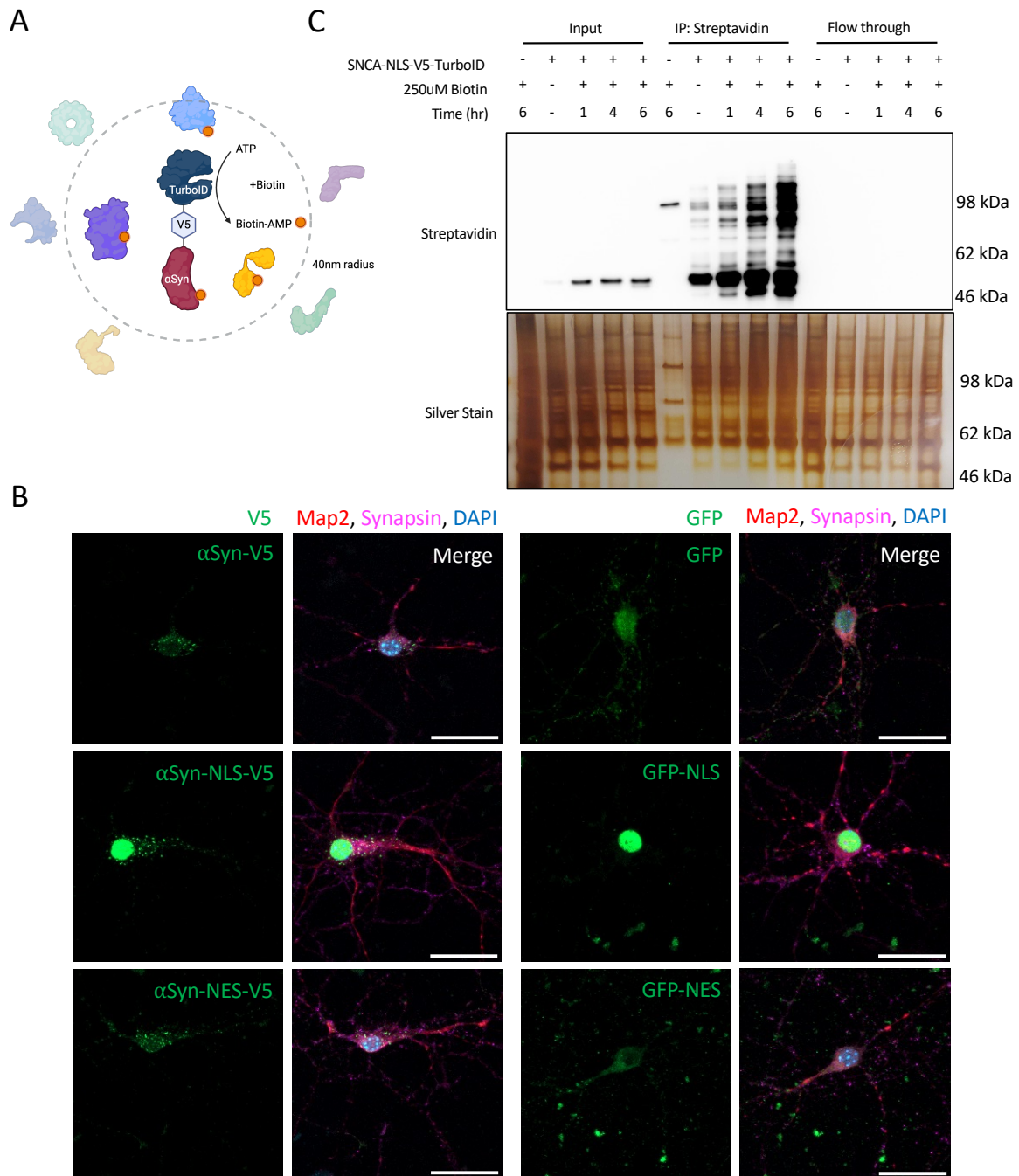


Figure 1: Using TurboID to identify α Syn subcellular-specific interactome. A) Graphic illustrating TurboID proximity labelling. B) Immunofluorescent staining of primary cortical neurons transduced with TurboID lentiviruses. Scale bars = 25 μ m. C) Proof-of-concept immunoprecipitation experiment demonstrates time-dependent biotinylation and efficient pull down of biotinylated proteins (upper) with corresponding silver stain (lower).

We analysed the three individual proximity interactomes and confirmed an enrichment of each respective subcellular compartment above background, supporting proper localization (**Fig 2a**). We also noted some overlap between the interactomes; however, many proteins were specific to each subcellular localization (**Fig 2b**). To help narrow hits from this proximity-based interactome with, we generated Flag-tagged knockin mice targeting α Syn to the nucleus (*Snca*^{NLS-Flag}, as published in Geertsma et al, 2022), to the cytoplasm (*Snca*^{NES-Flag}) and in its native localization (*Snca*^{Flag}) and performed Flag IPs coupled with mass spectrometry. Similar to the TurboID interactomes, we confirmed an enrichment of each respective subcellular compartment and noted some overlap between the subcellular interactomes although many were specific to the respective *Snca* subcellular localizations (**Fig 2c,d**). We next sought to determine if there were unified pathways enriched in both the *SNCA-NLS* or *Snca*^{NLS} interactomes to identify common threads for disease biology. Here, we noted significant enrichment of proteins involved in the RNA splicing and spliceosome pathway specific to the nuclear α Syn interactome (**Fig 2e**).

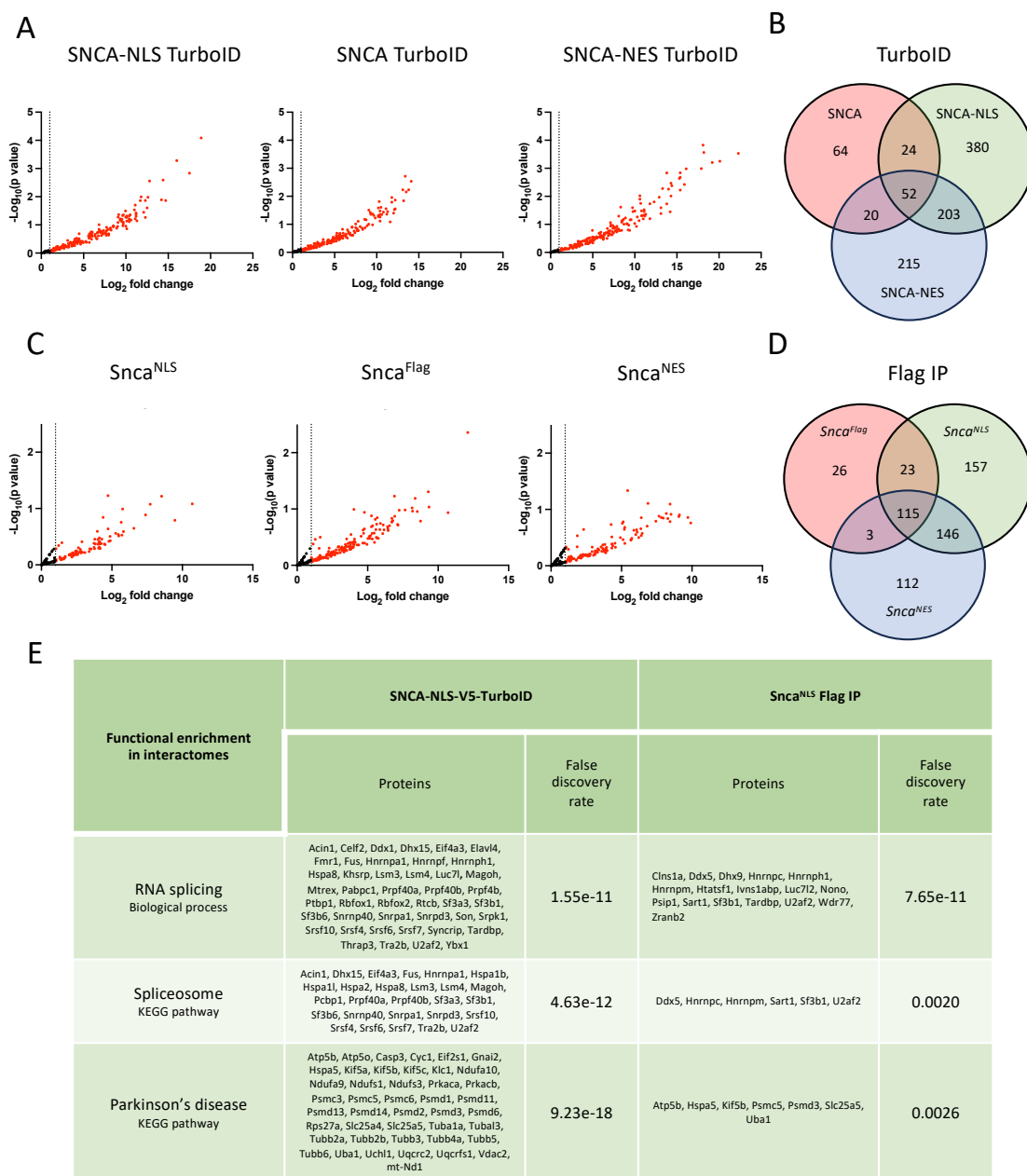


Figure 2: TurboID and Flag IP interactomes identify enrichment of splicing proteins interacting with nuclear α Syn. A) Volcano plots highlighting enriched proteins from SNCA-NLS-V5-TurboID (left), SNCA-V5-TurboID (middle), and SNCA-NES-V5-TurboID (right). Dashed vertical line denotes fold change > 1 (above wild-type) and red data points denote proteins included in subsequent analysis. Plotted volcano from $n=4$. B) Venn diagram of proteins identified from TurboID assay with overlap across the subcellular regions. C) Volcano plots highlighting enriched proteins from Snca^{NLS} (left), Snca^{Flag} (middle), and Snca^{NES} (right). Dashed vertical line denotes fold change > 1 (above wild-type) and red data points denote proteins included in subsequent analysis. Plotted volcano from $n=4$. D) Venn diagram of proteins identified from Flag IP with overlap across the subcellular regions. E) Functional enrichment analysis highlighting three prominent groups of proteins specific to nuclear α Syn.

Validation of TurboID using bimolecular fluorescent complementation and immunoprecipitation assays identifies consistent nuclear interaction between α Syn and Luc7l

To validate the interaction of α Syn with select proteins within the RNA-binding/spliceosome pathways, we first used bimolecular fluorescence complementation (BiFC). BiFC uses two non-fluorescent components of a fluorescent protein fused to either bait or prey proteins that, when in close proximity, will form a fluorescent complex to be detected through immunofluorescent assays (Nagai et al., 2002). This validates the proximity of proteins with α Syn and can provide additional insight into the subcellular topography of the interaction.

We chose top candidates enriched in the nuclear α Syn interactome including Luc7l, a U1 snRNP splicing complex protein required for 5' splice site selection, and Hnrnpk, an RNA binding protein involved in pre-mRNA processing (Matunis et al., 1992; Fortes et al., 1999). We chose other candidates based on preliminary results, including Luc7l2, Srsf4, Arpc5l, Eif4a1, Eif4a3, Tubb6, however these did not validate through BiFC nor V5 immunoprecipitation (data not shown). When co-expressing LUC7L fused with the N-terminus of an mVenus fluorescent protein (nV) along with α Syn fused with the C-terminus of an mVenus fluorescent protein (cV), we found positive fluorescent signal through BiFC. This was confirmed through confocal imaging and flow cytometry experiments, detecting exclusively nuclear colocalization, even in the absence of a NLS on α Syn (**Fig 3a-c**). Interestingly, the nuclear signal in the LUC7L-nV + SNCA-cV is distinct from that of SNCA-nV + SNCA-cV, suggesting specific subnuclear localization of the α Syn-LUC7L interaction. Using the *Snca*^{NLS} mice, we also noted colocalization between α Syn and Luc7l in neuronal nuclei throughout the brain with similar subnuclear localization of Luc7l (**Fig 3d**).

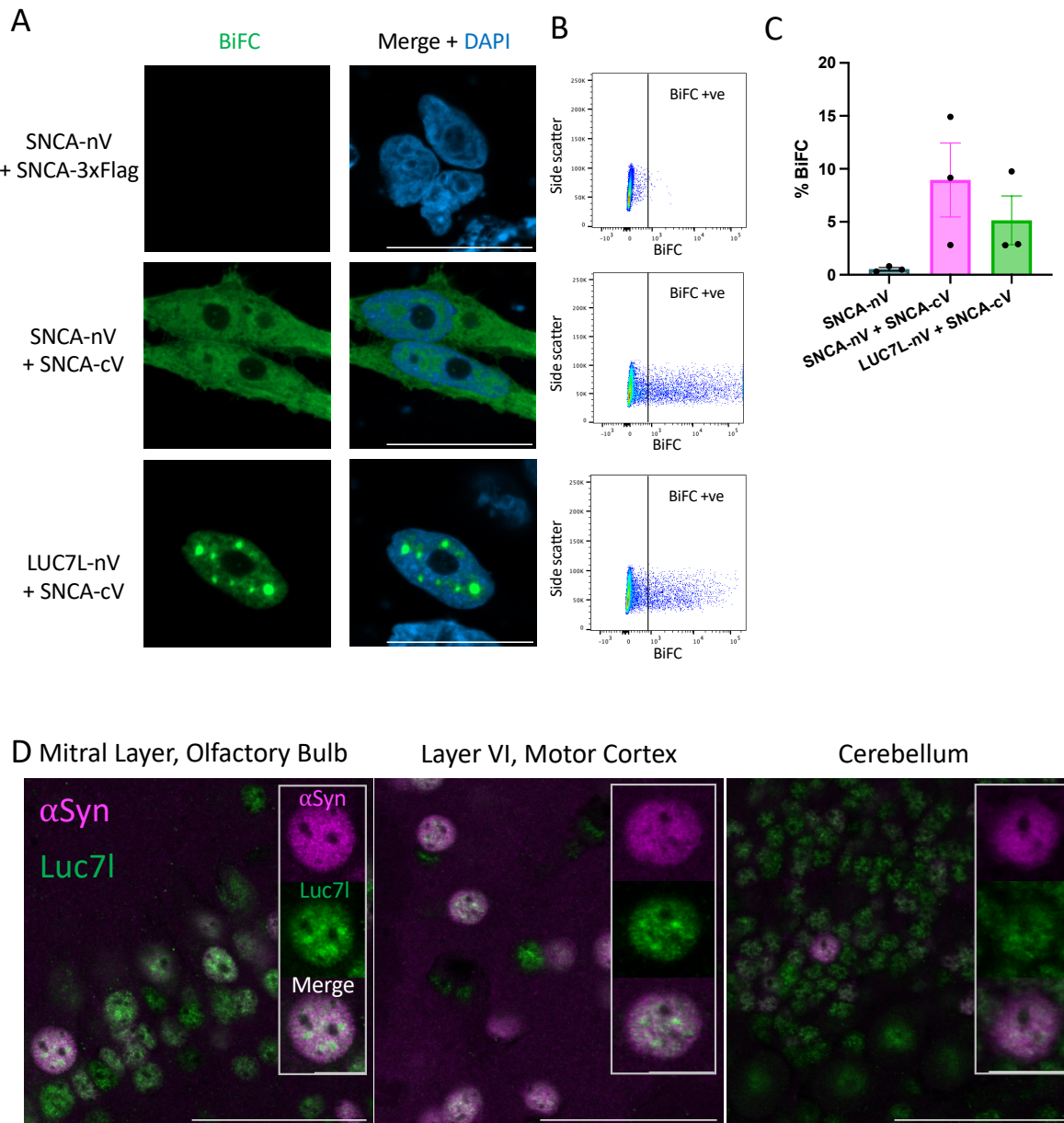
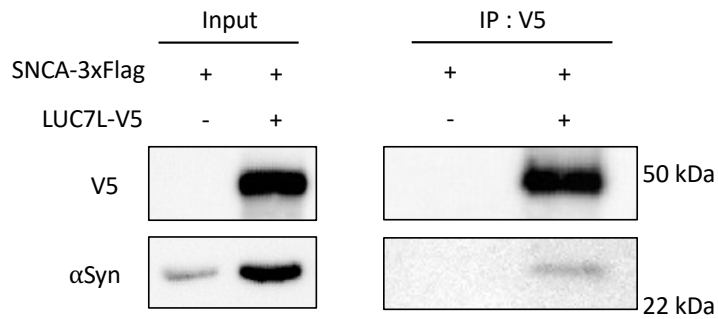


Figure 3: Bimolecular fluorescent complementation assay confirms nuclear-specific interaction with α Syn and colocalization is observed throughout the mouse brain. A) Bimolecular fluorescent complementation followed by immunofluorescent imaging in HeLa cells. Scale bars = 25 μ m. B) Bimolecular fluorescent complementation followed by flow cytometry plots highlighting BiFC+ signal in each condition. C) Quantification of BiFC+ cells from flow cytometry. Plotted n=3 mean with SEM. D) Immunofluorescent imaging of α Syn and Luc7l across *Snc*^{NLS} mouse brain. Scale bars = 75 μ m.

To increase our confidence in the LUC7L- α Syn interaction, we turned to the complementary approach of V5 immunoprecipitation of *LUC7L-V5* with *SNCA-3xFlag* from HEK293T cells (**Fig 4a**). We co-transfected HEK293T cells with either *LUC7L-V5* and/or *SNCA-3xFlag* then performed a V5 immunoprecipitation to identify proteins interacting with LUC7L. Indeed, we were able to detect α Syn, further confirming their interaction. To determine if this interaction exists in tissue with endogenous α Syn and Luc7l protein, we performed Luc7l immunoprecipitation on *Snca^{Flag}*, *Snca^{NLS}*, and *Snca^{NES}* mouse hemibrains (**Fig 4b**). Again, we could detect immunoprecipitated α Syn in the *Snca^{Flag}* and *Snca^{NLS}* samples, further supporting the robust nature of this interaction.

A



B

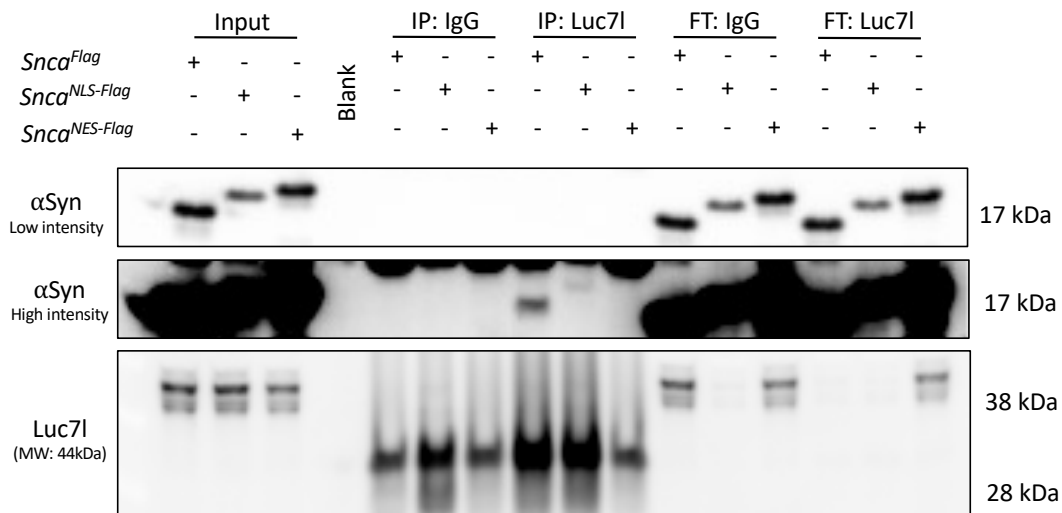


Figure 4: αSyn binds with LUC7L in HEK293T cells and mouse brain tissue. A) V5 immunoprecipitation from SNCA-3xFlag and LUC7L-V5 co-overexpression in HEK293T cells. B) Immunoprecipitation of LUC7L conjugated to IgG beads from the *Snca*^{Flag}, *Snca*^{NLS}, and *Snca*^{NES} brain lysates.

LUC7L overexpression stabilizes α Syn protein levels

When analyzing the input fractions from our initial immunoprecipitation assays, we noted that α Syn protein levels increase upon LUC7L overexpression (**Fig 5a**). We note a ~50% increase in LUC7L following α Syn overexpression and an >100% increase in α Syn following LUC7L overexpression in HEK293T cells. Since these cells have lower levels of endogenous α Syn protein, we turned to the more physiologically-relevant primary cortical neurons. We generated LUC7L-V5 and mCherry (control) lentiviruses and transduced wild-type primary cortical neurons at an MOI of 3. After 9 days, we ran whole cell lysates on a western blot and found that LUC7L overexpression similarly increases endogenous α Syn levels (**Fig 5b**).

Since α Syn has been shown to accumulate in disease, we next sought to explore whether LUC7L protein levels are elevated in PD. Using immunofluorescent staining on PD post-mortem brain tissue, we noted a modest increase in LUC7L fluorescent intensity in PD brains relative to non-neurodegenerative controls, although more replicates are needed for accurate quantification (**Fig 5c**). These results further support the nuclear interaction between LUC7L and α Syn and suggests a possible mechanism of disease whereby increased LUC7L can lead to a stabilization of α Syn, and, over decades, may be potentiating its nuclear accumulation and/or aggregation.

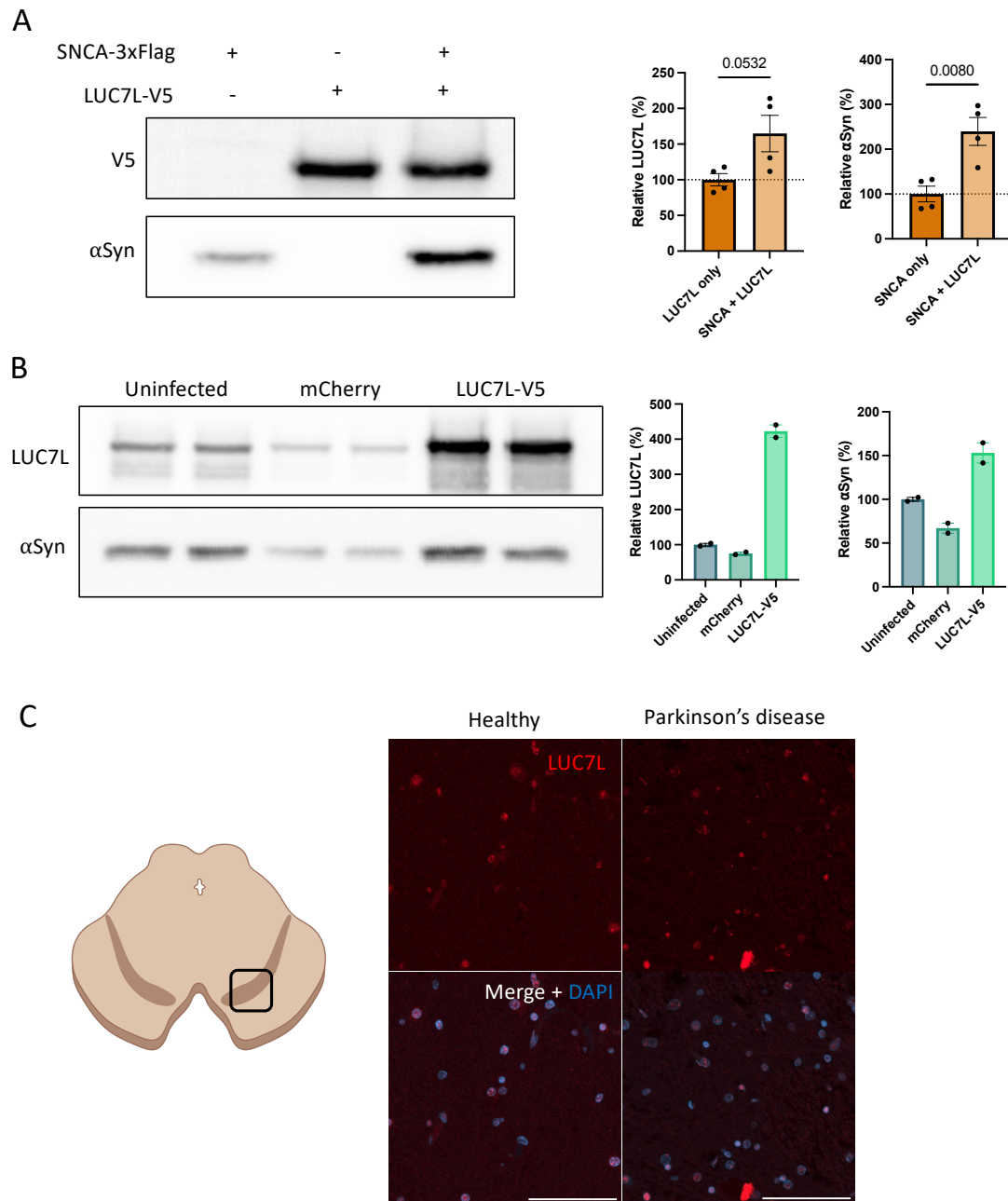


Figure 5: LUC7L overexpression stabilizes α Syn protein, and LUC7L is elevated in PD midbrain. A) Western blot of whole cell lysates from co-overexpression of SNCA-3xFlag and LUC7L-V5 (left) with corresponding quantification of LUC7L (middle), and α Syn (right). Plotted n=4 mean with SEM and analyzed with parametric unpaired t test, p values displayed on the graphs. B) Western blot of whole cell lysates from wild-type primary cortical neurons transduced with LUC7L-V5 or mCherry lentivirus (left) with corresponding quantification of LUC7L (middle) and endogenous α Syn (right). Plotted preliminary n=2 mean with SEM. C) Depiction of sampled brain region (left) with immunofluorescent staining of LUC7L from PD post-mortem tissue (right).

Discussion

The mechanisms underlying α Syn toxicity, including its nuclear toxicity, have been difficult to uncover. To begin to parse out these mechanisms, we performed an unbiased proximity labelling screen using the TurboID enzyme to identify proteins that interact with α Syn both within and outside the nucleus. The α Syn interactome has been previously explored and α Syn was found to interact with proteins involved in mRNA metabolism in neurons (Chung et al., 2017; Griffin et al., 2023). This study builds upon these existing datasets by homing in on the nuclear-specific interactome to provide refinement on a subcellular level. In this study, we overexpressed *SNCA-NLS-V5-TurboID*, in addition to other control or comparative conditions, in *Snca*^{KO} primary cortical neurons. We identified significant enrichment of spliceosome proteins and highlight Luc7l as a strong interacting protein. There is little known about Luc7l in the context of neurodegenerative disorders; previous studies on the protein have surrounded its involvement in the blood disorder α -thalassemia (*Luc7l* truncation; Tufarelli et al., 2003). Interestingly, of all three proteins in the Luc7 family (Luc7l, Luc7l2, and Luc7l3), Luc7l has the highest protein level in the midbrain (Jourdain et al., 2021). Luc7l is a nuclear protein, and we found an exclusively nuclear interaction with α Syn that was distinct from the typical subnuclear localization of α Syn alone. We corroborated our proximity-based findings with co-immunoprecipitation of α Syn and Luc7l.

Serendipitously, we discovered that overexpressing LUC7L led to a higher degree of α Syn overexpression in HEK293T cells and was confirmed to increase endogenous α Syn in wild-type primary cortical neurons transduced with LUC7L lentivirus. This has larger implications for disease, as increased α Syn stabilization can lead to its accumulation and aggregation. Indeed, when examining PD post-mortem brain slices, we noted more LUC7L

fluorescent intensity, indicating a potential increased abundance of LUC7L in disease. These findings suggest that LUC7L could be contributing to disease by stabilizing nuclear α Syn and leading to its accumulation which, over time, could also lead to increased α Syn aggregation and toxicity. α Syn is also a small protein than can passively diffuse through the nuclear pore, however LUC7L could be maintaining its nuclear localization, which in turn could lead to an overcompensation of total α Syn protein.

Although we are the first to explore the nuclear-specific α Syn interactome in this manner, this nuclear interactome shares many enriched proteins to a previous study that performed proximity labelling of overexpressed α Syn in rat primary cortical neurons (Chung et al., 2017). Although they did not use a nuclear localization tag on α Syn, the protein does still accumulate in the nucleus upon overexpression, therefore we can compare these datasets to glean insight into the interactions following different conditions. Both investigations found an enrichment for RNA-binding proteins, including many in the Hnrnp and Ddx family, and in protein biosynthesis, including several Eif proteins. Although they did not identify Luc7l as an interactor of α Syn, they did identify an enrichment of Tia1, which has been shown to have similar mRNA binding to Luc7l along with Hnrnpk, suggesting a potential complex of proteins with which α Syn is interacting in disease (Daniels et al., 2021). Understanding the role of α Syn protein-protein interactions will be imperative in the development of novel therapeutics that can specifically target these interactions, with the ultimate goal of developing disease-modifying therapeutics.

Materials and Methods

Generating the TurboID plasmids

The *SNCA-V5-TurboID*, *SNCA-NLS-V5-TurboID*, and *SNCA-NES-V5-TurboID* plasmids were ordered through Twist Biosciences by inserting the sequencings into their pTwist Lenti SFFV Puro WPRE vector with a BamHI site upstream of *SNCA* and XhoI site downstream of V5. The *GFP*, *GFP-NLS*, and *GFP-NES* sequences were ordered from Twist Biosciences flanked by BamHI and XhoI sites. We used these restriction enzymes with the *SNCA-V5-TurboID* plasmid to knock in the GFP controls into the TurboID backbone.

Generating the bimolecular fluorescence complementation plasmids and LUC7L-V5 plasmid

Genes of interest were ordered from the University of Ottawa Genome Editing and Molecular Biology (GEM) facility orfeome library in pENTR221 vectors. We used LR cloning to insert the genes of interest into pBABE-nVenus-DEST, pBABE-DEST-nVenus, or pBABE-DEST-cVenus (for BiFC) or pLEX307-DEST-V5 (for V5-IP) plasmids following LR Clonase kit instructions (ThermoFisher 11-709-020). Briefly, 50 ng of pENTR221 plasmid and 75 ng of destination plasmid were combined with 1 µl LR Clonase and TE buffer then incubated at room temperature for 1 hour prior to transformation into Stab13 chemically competent cells. All plasmids were sequenced to confirm successful cloning.

Lentivirus generation

On day 0, low passage HEK293T cells were plated onto five 15 cm² at ~40% confluency. The following day, the cells were transfected with a mixture of equal weight ratios of the TurboID plasmid, psPAX2, and pMD2-G to a total mass of 25,500 ng; 2 mL OptiMEM (ThermoFisher

31985070), and 102 μ L ePEI (uOttawa BioBar) per 15 cm² plate. After 4 hours, the full media was changed. On day 1, the full media was changed and replaced with 14 mL DMEM complete (10% fetal bovine serum, 1X antibiotic-antimycotic). On day 2, the media was collected and stored at 4 °C and replaced with 14 mL DMEM complete. On day 3, then media was collected and combined with day 2 media collection then filtered through 0.45 μ m vacuum filter. This filtered media was centrifuged at 20,000 rpm for 2 hours at 4 °C, then the supernatant was discarded, and the pellet resuspended in sterile 1X PBS and stored at -80 °C.

Generating $Snca^{Flag}$, $Snca^{NLS}$, and $Snca^{NES}$ mice

Mouse engineering: Generation of the $Snca^{NLS}$ mice was previously described and they are available from the Jackson Laboratory (036763). The $Snca^{Flag}$ and $Snca^{NES}$ mice were generated at the same time using the same protocol (Geertsma et al., 2022). Briefly, Cas9 protein was complexed to a sgRNA targeting the 3' locus of $Snca$ (sgRNA target sequence:

5'-TTGGTAGCCTTCCTAATATC-3'); and, together with a single-stranded

oligodeoxynucleotide (ssODN) repair template ($Snca^{Flag}$:

5'-CACTGTGAAGCAGACAGTTGATATCTGTCACTTCACTGACAAGGCATGCTGTTATT
ATTTTCTTTTTCTGATATTAGGAAGGCTACCAAGACTATGAGCCTGAAGCCGACTACA
AGGACGACGACGACAAGTAAGAATGTCATTGCACCCAATCTCCTAAGATCTGCCGGC
TGCTCTTCCATGGCGTACAAGTGCTCAGT-3'; $Snca^{NES}$:

5'- ATATCTGTCACTTCACTGACAAGGCATGCTGTTATTATTTTCTTTTTCTGATATTAGG
AAGGCTACCAAGACTATGAGCCTGAAGCCGCCCTGCAGAAGAAGCTGGAGGAGCTG
GAGCTGGACGAGGACTACAAGGACGACGACGACAAGTAAGAATGTCATTGCACCCA
ATCTCCTAAGATCTGCCGGCTGCTCTTCCA-3'; IDT Ultramer), were injected and

implanted into pseudo-pregnant FVB female mice (Rousseaux et al., 2018). Three founder mice were generated and backcrossed onto a ~99.75% pure C57Bl6/J background, before being expanded onto a mixed C57Bl6/J:C57Bl6/NCrI background. One line from each genotype was selected for subsequent characterization.

Genotyping: A small (~1mm) tail sample is digested prior to PCR amplification using primers outside of the sequence covered by the ssODN used for the initial mouse line. Forward:

5'-TTTTATCTGATTGAAATGATGAGC-3'; Reverse: 5'-ATGACTGGGCACATTGGAA-3'.

PCR protocol: 95 °C for 2 minutes, (95 °C for 30 seconds, 56 °C for 30 seconds, 72 °C for 30 seconds) repeated for 35 cycles, 72 °C for 5 minutes. Wildtype allele: 225 bp; *Snca*^{Flag} allele: 249 bp; *Snca*^{NLS} allele: 273 bp; *Snca*^{NES} allele: 288 bp.

Mouse husbandry

All mice were housed with up to 5 mice per cage on a 12-hour light-dark cycle. Mice were fed *ad libitum* and all husbandry was performed by the uOttawa Animal Care and Veterinary Services staff. All animal work was performed under the approved breeding (CMMb-3904) and dissection (CMMe-4030) protocols approved under the uOttawa Animal Care Committee.

Culturing primary cortical neurons

One male and one female mouse were paired for 3 days before the male was removed. The female mouse was weighed at time of pairing and if after 10 days she gained >10% body weight, it was deemed a successful pregnancy. At 13 days after pairing, the pregnant mouse was sedated with 120 mg/kg Euthanyl (DIN00141704) then decapitated. Embryos at E13.5-E15.5 were removed, and their cortex was isolated in a separate tube in 700 µL Hank's balanced salt solution

(HBSS; Millipore Sigma H9394) then pooled. 20 μ L trypsin (Sigma Aldrich T4549) was added per embryo, and this was incubated at 37 °C for 20 minutes on a rotator. Next, titurate solution slowly and carefully with ‘Solution A’ [2.75 mL unsupplemented neurobasal (ThermoFisher 21103049), 150 μ L trypsin inhibitor (chicken egg white at 10 mg/mL), 100 μ L DNase (Qiagen 79254)] then centrifuge at 2500x g for 5 minutes at 4 °C. Remove supernatant and titurate pellet slowly and carefully with ‘Solution B’ (2.55 mL unsupplemented neurobasal, 200 μ L trypsin inhibitor, 250 μ L DNase) then centrifuge at 2500x g for 5 minutes at 4 °C. Remove supernatant and resuspend pellet in 10 mL neurobasal complete [neurobasal media, 1X B-27 (ThermoFisher 17504044), 1X N-2 (ThermoFisher 17502-048), 1X Pen-strep (Cytiva SV30010)]. Neurons were then plated in neurobasal complete and incubated at 37 °C for 4 days before any media change. Downstream applications detailed below.

Staining and imaging primary cortical neurons

Snca^{KO} primary cortical neurons infected and plated in a 24-well dish on coverslips were incubated at 37 °C for 4 days prior to a full media change with neurobasal complete then an additional 3 days of incubation. The media was then removed, and neurons were rinsed with 1X PBS x2 washes then fixed in 10% buffered formalin (ThermoFisher SF100-4) for 10 minutes at room temperature. Neurons were rinsed with 1X PBS x2 then incubated in blocking buffer (10% horse serum (Sigma Aldrich H0146-5ML), 0.5 % triton X-100 (Sigma Aldrich T8787-100ML) in 1X PBS) for 1 hour at room temperature. Neurons were then incubated in primary antibody solution [Map2 (RRID:AB_2138153), V5 (RRID:AB_2938590), Synapsin (RRID:AB_887805) at 1:1,000] diluted in blocking buffer overnight at 4°C. The following day, the neurons were incubated in secondary antibody [AlexaFluor-594 (RRID:AB_2534079), and -647

(RRID:AB_2535866) at 1:500, DAPI (Millipore Sigma D9542) at 1:1,000] diluted in blocking buffer, then mounted on #1.5 coverslips with 4 μ L mounting medium (Agilent, S302380-2).

Neurons were imaged with a confocal microscope (LSM800 AxioObserverZ1).

TurboID assay

Primary cortical neurons were infected at time of plating and incubated at 37 °C for 4 days prior to a full media change with biotin-free neurobasal complete [wash streptavidin agarose beads (ThermoFisher S951) in sterile water x2 then incubate 1 mL beads per 50 mL neurobasal complete on a rotator at 4 °C overnight. Pellet agarose beads and save biotin-free media] for 3 days. Add 250 μ M biotin (Sigma Aldrich B4639) or water and incubate at 37 °C for 10 minutes then place cells on ice and rinse with ice cold 1X PBS for 4 washes. Neurons were then lysed in 300 μ L TurboID lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% w/v sodium dodecyl sulfate, 0.5% w/v sodium deoxycholate, 1% triton X-100 at pH 7.5) and scraped off the plate then incubated on ice for 20 minutes. Lysate was centrifuged at 13,000x g for 10 minutes at 4 °C and the supernatant was saved in a new tube.

Streptavidin immunoprecipitation

To this lysate, add 50 μ L streptavidin magnetic beads [(ThermoFisher 88817) washed with TurboID lysis buffer x2] and incubated on a rotator overnight at 4 °C. The flow through was then removed and the beads were washed with 1 mL TurboID lysis buffer x3 washes. For mass spectrometry, see subsequent protocol titled ‘Streptavidin immunoprecipitation for mass spectrometry’. For a western blot, add 50 μ L 2X Laemmli, and heat the beads to 95 °C for 7 minutes then follow the protocol below.

Western blot

Samples were run on a 4-15% Bolt MES-PAGE for 75 minutes then transferred to a 0.2 μ m nitrocellulose membrane for 90 minutes at 330 mA. The membrane was blocked in 10% milk (diluted in 1X TBS-T) for 1 hour at room temperature then incubated in primary antibody [diluted in 2% bovine serum albumin; V5 (RRID:AB_159313), LUC7L (RRID:AB_2878346), α Syn (RRID:AB_2537217 for human α Syn or RRID:AB_398108 for mouse α Syn) at 1:1,000] overnight at 4 °C on a rocker. The following day, the blot was incubated in horseradish peroxidase-conjugated secondary antibody (RRID:AB_2340770 for mouse or RRID:AB_10015282 for rabbit antibodies, both used at 1:10,000) diluted in 10% milk for 1 hour at room temperature. The membrane was then rinsed with ECL Clarity solution (Bio-Rad 1705061), imaged with a GE ImageQuant LAS4000, and analyzed with ImageLab (version 6.1).

Streptavidin immunoprecipitation for mass spectrometry

To the TurboID lysate, 50 μ L streptavidin magnetic beads (washed with TurboID lysis buffer x2) was added and incubated on a rotator overnight at 4 °C. The flow through was then removed and the beads were washed with 1 mL TurboID lysis buffer x3 washes then 1 mL 50 mM ammonium bicarbonate pH 8 x2. The beads were quickly spun, and any additional liquid was removed prior to adding 1 μ g trypsin (Promega VA900A). These were incubated on a rotator at 37 °C overnight. The following day, an additional 1 μ g of trypsin was added to the bead solution and incubated on a rotator at 37 °C for 3 hours. The IP flow through was then saved in a new tube and the beads were washed with 100 μ L water x2 and that flow through was added to the IP flow through. 50% formic acid was added to a final concentration of 2% then the samples were dried on a speed vac

overnight. Samples were reconstituted in 20 µl 1% formic acid and submitted to the Ottawa Hospital Research Institute Proteomics Core.

Flag immunoprecipitation for mass spectrometry

Primary cortical neurons from *Snca*^{Flag}, *Snca*^{NLS-Flag}, and *Snca*^{NES-Flag} mice were cultured for 7 days in neurobasal complete media. On day 7, the neurons were rinsed with 1 mL 1X PBS x3 then lysed in 300 µL Flag lysis buffer (25 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol). Lysate was transferred to a new tube and incubated on ice for 20 minutes then centrifuged at 13,000x g for 10 minutes at 4 °C. The supernatant was moved to a new tube and 50 µL Flag M2 beads [(RRID:AB_2637089) washed in Flag lysis buffer x2] were added and incubated on a rotator for 1 hour at 4 °C. The flow through was then removed and the beads were washed with 1 mL Flag lysis buffer x3 washes then 1 mL 50 mM ammonium bicarbonate pH 8 x2. The beads were quickly spun, and any additional liquid was removed prior to adding 1 µg trypsin (Promega VA900A). These were incubated on a rotator at 37 °C overnight. The following day, an additional 1 µg of trypsin was added to the bead solution and incubated on a rotator at 37 °C for 3 hours. The IP flow through was then saved in a new tube and the beads were washed with 100 µL water x2 and that flow through was added to the IP flow through. 50% formic acid was added to a final concentration of 2% then the samples were dried on a speed vac overnight. Samples were reconstituted in 20 µl 1% formic acid and submitted to the Ottawa Hospital Research Institute Proteomics Core.

Mass spectrometry

Proteomics analysis was performed at the Ottawa Hospital Research Institute Proteomics Core Facility (Ottawa, Canada). Peptide extracts were concentrated by Vacufuge (Eppendorf) and purified with C18 Ziptips (Millipore Sigma). LC-MS/MS was performed using a Dionex Ultimate 3000 RLSC nano HPLC (Thermo Scientific) and Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific). MASCOT software (version 2.8.2, Matrix Science, UK) was used to infer peptide and protein identities from the mass spectra. The observed spectra were matched against mouse sequences from SwissProt (version 2022_05) and against an in-house database of common contaminants and custom constructs. The results were exported to Scaffold (version 5.3.2, Proteome Software, USA) for further validation and viewing.

Bimolecular fluorescence complementation assay with confocal imaging

HeLa cells were plated on 24-well coverslips [(VWR 100500-834) acid washed in 2 M HCl for 5 hours then coated in 0.05 mg/mL poly-d-lysine overnight at 37 °C] and the following day transfected with 250 ng of each BiFC plasmid with 2 µL ePEI and 50 µL OptiMEM. After 72 hours, the cells were rinsed with 1X PBS x2 then fixed in 10% buffered formalin (ThermoFisher SF100-4) for 10 minutes at room temperature. Cells were rinsed with 1X PBS x2 then incubated in blocking buffer (10% horse serum + 0.5% triton X-100 in 1X PBS) with DAPI 1:1000 for 1 hour at room temperature. The cells were washed then mounted on Superfrost™ plus slides (Fisher Scientific 12-550-15) with 4 µL fluorescent mounting medium (Agilent, S302380-2). Cells were imaged with a confocal microscope (LSM800 AxioObserverZ1).

Bifluorescence molecular complementation assay with flow cytometry

HeLa cells were plated on 12-well plate and the following day transformed with 250 ng of each BiFC plasmid with 2 μ L ePEI and 50 μ L OptiMEM. After 72 hours, the media was removed, and the cells were trypsinized with 0.25% trypsin-EDTA for 5 minutes at 37 °C then quenched in FACS buffer (1 mM EDTA, 2% FBS in 1X PBS) and pipetted up/down x5. The cell suspension was run through the BD LSRFortessa from the University of Ottawa Flow Cytometry and Virology Core.

Mouse tissue staining

Previously generated mouse brain tissue slices at 40 μ m were stained as follows (Geertsma et al., 2022): tissue was washed in 1X PBS x2 then blocked in IF blocking buffer (5% cosmic calf (Cytiva SH30087.03), 0.5% triton X-100 in 1X PBS) for 1 hour at room temperature then incubated in primary antibody solution [α Syn (RRID:AB_398108), Luc71 (RRID:AB_2878346) at 1:1,000] overnight. The following morning, the tissue was incubated in secondary antibody solution [AlexaFluor-488 (RRID:AB_2534069), -647 (RRID:AB_2535812) at 1:500, and DAPI (Millipore Sigma D9542) at 1:1,000] for 2 hours at room temperature then the tissue was washed in 1X PBS and mounted on superfrost plus slides. The tissue was covered with fluorescent mounting medium and #1.5 coverslips (VWR 100500-818) before confocal imaging.

LUC7L-V5 immunoprecipitation from HEK293T cells

HEK293T cells cultured in a 6-well dish were transfected with 1,500 ng of *SNCA-3xFlag* or 1,000 ng of *LUC7L-V5* or both along with 10 μ L ePEI and 200 μ L OptiMEM. Media was changed after 4 hours then the cells were left for 72 hours. Next, all media was removed from the

cells then lysed in the plate with 300 μ L Flag lysis buffer. Lysates were incubated on ice for 20 minutes then centrifuged at 13,000x g for 10 minutes at 4 °C. 20 μ L of V5-trap beads (Bulldog Bio V5TMA020) were washed in 1 mL Flag lysis buffer x2 then resuspended in 20 μ L of Flag lysis buffer and added to 100 μ L of lysate supernatant. This was incubated on a rotator at 4 °C for 3 hours. Next, the beads were washed with Flag lysis buffer x3 before eluting in 30 μ L 2X Laemmli at 95 °C for 5 minutes.

Luc7l immunoprecipitation from mouse brain

Frozen mouse hemibrains were thawed on ice then homogenized in 10 mL Flag lysis buffer with a dounce homogenizer. Lysate was incubated on ice for 10 minutes then centrifuged at 21,130x g for 30 minutes at 4 °C. 100 μ L supernatant was used for immunoprecipitation and the remaining supernatant was stored at -80 °C for future use. To the supernatant, either 1 μ g rabbit IgG (Cedarlane 20009-1-200) or 1 μ g Luc7L (Proteintech 17085-1-AP) was added and incubated on a rotator at 4 °C for 1 hour. 20 μ L Protein G Dynabeads (ThermoFisher 10004D) were washed in Flag lysis buffer x2 then added to each sample and incubated on a rotator at 4 °C for 3 hours. Beads were washed in Flag lysis buffer x2 then eluted in 30 μ L 2X Laemmli and heated to 80 °C for 10 minutes.

LUC7L- α Syn stability assays

Figure 5A: HEK293T cells cultured in a 6-well dish were transfected with 500 ng of *SNCA-3xFlag* or *LUC7L-V5* or both along with 10 μ L ePEI and 200 μ L OptiMEM. Media was changed after 4 hours then the cells were incubated for 72 hours. Next, cells were washed with 1X PBS x2 then lysed in the plate with 300 μ L Flag lysis buffer. Whole cell lysate was moved to

a new tube and 4X Laemmli was added to a final concentration of 1X then heated to 95 °C for 7 minutes.

Figure 5B: Wild-type primary cortical neurons were cultured (see ‘Primary cortical neuron’ protocol above) and infected with 3 MOI mCherry or LUC7L-V5 lentivirus at day 0. A full media change was performed on day 7, then on day 9 the neurons were rinsed in 1X PBS then lysed in Flag lysis buffer. Lysate was moved to a new tube and 4X Laemmli was added to a final concentration of 1X then heated to 95 °C for 7 minutes.

Staining human post-mortem brain tissue

All human tissue was collected in accordance with Institutional Review Board-approved guidelines. Paraffin-embedded human samples were obtained from the Neuropathology Service at Brigham and Women’s Hospital in Boston, MA and from archived autopsy specimens in the Department of Pathology and Laboratory Medicine of The Ottawa Hospital in Ottawa, ON. Ethics approval was obtained from the Ottawa Health Science Network Research Ethics Board (#20120963-01H).

This midbrain tissue was sectioned 5 µm and deparaffinized in serial baths of 100% xylenes for 20 minutes x2, 100% ethanol for 5 minutes, 95% ethanol for 5 minutes, 80% ethanol for 5 minutes, 60% ethanol for 5 minutes, then 1X PBS for 1 minute x3. Next, the tissue underwent antigen retrieval in 1X Tris-EDTA pH 9 for 20 minutes at 95 °C. Tissue was washed in 1X PBT (0.1% triton X-100 in 1X PBS) then incubate in blocking buffer (5% cosmic calf serum, 0.5% triton X-100) for 2 hours at room temperature then primary antibody solution [LUC7L (RRID:AB_2878346), DAPI (Millipore Sigma D9542) at 1:1,000] overnight at 4 °C. The following day, tissue was washed in 1X PBT then incubated in secondary antibody solution

for 2 hours at room temperature. Tissue was then washed in 1X PBT then 1X PBS, then underwent autofluorescent elimination as per solution instructions [(Millipore Sigma 2160) incubate in 70% ethanol for 5 minutes, then autofluorescence eliminator for 2 minutes, then wash in 70% ethanol for 1 minute x3]. Tissue was then covered with fluorescent mounting medium and #1.5 coverslips.

Statistical analysis

Statistical analysis for Figure 2 was performed using R Studio. All other statistical analysis was performed with GraphPad Prism (version 10.2.1) using the appropriate statistical test, either a Student's t-test for simple comparisons or one-way ANOVA. Statistical test used, sample sizes, and p-values are delineated in each figure legend. All graphs plot the mean with the standard error of the mean (SEM).

Acknowledgements

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expertise. Fig. 1a and 5c were generated, in part, using Biorender.com. All histograms were generated with Prism 10 (version 10.2.2).

Chapter 4: A topographical atlas of α Syn dosage and cell type-specific expression in adult mouse brain and peripheral organs

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Statement of author contribution

This manuscript uses the *Sncα^{NLS}* mice as a reporter for αSyn protein localization throughout the mouse central nervous system (CNS), retina, and gut. Due to αSyn having predominantly presynaptic localization, immunostaining for the protein results in a diffuse staining pattern, making it difficult to identify the types of cells that express αSyn. Here, we find αSyn protein highly correlates with its RNA expression, further validating fluorescent *in situ* hybridization mapping of *Sncα* expression. In the CNS, we found that αSyn is highly expressed in neurons in the olfactory bulb, SNc, and dorsal motor nucleus of the vagus in the brain and the upper laminae of the dorsal horn throughout the spinal cord. Surprisingly, αSyn was relatively absent from ChAT-positive motor neurons in the spinal cord, however, was expressed to some degree in all enteric neurons. Together, this atlas provides invaluable insight into the localization of αSyn throughout the mouse that will enable better interpretation of models studying αSyn both in healthy and disease states. This quantitative cellular topography of αSyn provides insight into the selective vulnerability of neurons seen in PD and other α-synucleinopathies.

Haley Geertsma, Zoe Fisk, Michael Henderson and Maxime Rousseaux conceived the study. **Haley Geertsma**, Zoe Fisk, Lillian Sauline, and Kevin Kurgat performed all brain tissue acquisition, staining, imaging. **Haley Geertsma** performed all spinal cord and retina acquisition, staining, and imaging, and Zoe Fisk performed all analysis of these tissues. **Haley Geertsma** performed all gut acquisition and Alice Prigent performed all gut staining and imaging. **Haley Geertsma**, Zoe Fisk, Lillian Sauline and Dr. Alice Prigent, Dr. Michael Henderson, and Dr. Maxime Rousseaux analyzed data. **Haley Geertsma**, Dr. Michael Henderson and Dr. Maxime Rousseaux wrote the paper.

A topographical atlas of α -synuclein dosage and cell type-specific expression in adult mouse brain and peripheral organs

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Abstract

Parkinson's disease (PD) is the second most common neurodegenerative disease worldwide and presents pathologically with Lewy pathology and dopaminergic neurodegeneration. Lewy pathology contains aggregated α -synuclein (α Syn), a protein encoded by the *SNCA* gene which is also mutated or duplicated in a subset of familial PD cases. Due to its predominant presynaptic localization, immunostaining for the protein results in a diffuse reactivity pattern, providing little insight into the types of cells expressing α Syn. As a result, insight into α Syn expression-driven cellular vulnerability has been difficult to ascertain. Using a combination of knock-in mice that target α Syn to the nucleus (*Snca^{NLS}*) and *in situ* hybridization of *Snca* in wild-type mice, we systematically mapped the topography and cell types expressing α Syn in the mouse brain, spinal cord, retina, and gut. We find a high degree of correlation between α Syn protein and RNA levels and further identify cell types with low and high α Syn content. We also find high α Syn expression in neurons, particularly those involved in PD, and to a lower extent in non-neuronal cell types, notably those of oligodendrocyte lineage, which are relevant to multiple system atrophy pathogenesis. Surprisingly, we also found that α Syn is relatively absent from select neuron types, e.g., ChAT-positive motor neurons, whereas enteric neurons universally express some degree of α Syn. Together, this integrated atlas provides insight into the cellular topography of α Syn, and provides a quantitative map to test hypotheses about the role of α Syn in network vulnerability, and thus serves investigations into PD pathogenesis and other α -synucleinopathies.

Introduction

Parkinson's disease (PD) and related dementias exist along a clinical and pathological continuum, sharing a common protein pathology. α -synuclein (α Syn) undergoes aggregation and is thought to contribute to disease pathogenesis in several neurodegenerative diseases (i.e. PD, Lewy body dementia [LBD], multiple system atrophy [MSA]), collectively termed α -synucleinopathies (Hijaz & Volpicelli-Daley, 2020; Reddy & Dieriks, 2022). α Syn accumulates in proteinaceous structures called Lewy bodies and Lewy neurites (collectively: Lewy pathology) except for MSA where it accumulates in glial cytoplasmic inclusions (GCIs; Papp et al., 1989; Spillantini et al., 1997). Mutations and copy number gains in the gene encoding α Syn, *SNCA*, can also cause PD (Nishioka et al., 2006; Singleton et al., 2003). Thus, the convergence of pathology and genetics place α Syn at the center of pathogenesis for these disorders. Despite its linkage to PD over 25 years ago, less is known about the physiological role of α Syn and how it transitions from a native, functional form to a pathogenic state (Maroteaux et al., 1988). This transition and its cell-selective formation of pathology are important not only to neuronal α -synucleinopathies, such as PD and LBD, but also to the oligodendroglialopathy, MSA (Wenning et al., 2008). Normally, α Syn is found at the presynaptic membrane, and to lesser extents in the nucleus, mitochondria, and cytosol (Huang et al., 2011; Kahle, Neumann, Ozmen, Müller, et al., 2000; Miraglia et al., 2018; Specht et al., 2005; Wang et al., 2019). In disease conditions, α Syn mis-localizes from the membrane to cytosolic compartment where it adopts beta-sheet fibrillar conformations (Cremades et al., 2012; de Oliveira & Silva, 2019; Iljina et al., 2016). How initial misfolding events are linked to the ultimate formation of Lewy pathology remains unclear.

Increasing evidence suggests that expression levels of α Syn, as well as posttranslational modifications, conformational changes, and cellular milieu all play a role in modulating its aggregation (Flagmeier et al., 2016; Just et al., 2022; Nishioka et al., 2006; Singleton et al., 2003). Biochemical and biophysical data have begun to establish key modifications and conformations that lead to α Syn accumulation and aggregation (Fan et al., 2023; Li et al., 2022; Manzanza et al., 2021). Moreover, increasing data suggest that misfolded α Syn may act in a prionoid manner, spreading from one cell to another, initiating aggregate formation (Dadgar-Kiani et al., 2022; Vargas et al., 2019). This process requires both a template and substrate and therefore is dependent on α Syn expression in the target cell (Luk, Kehm, Zhang, et al., 2012a; Masuda-Suzukake et al., 2013; Vasili et al., 2022). Yet, topographical measurement of α Syn expression has been relatively understudied to date. This may be in part due to the limited tools available to glean meaningful insight at sufficient resolution. Since α Syn is largely localized to synapses, immunodetection in healthy individuals or model organisms results in presynaptic staining, making it difficult to attribute expression to any particular cell. RNA detection methods such as fluorescence *in situ* hybridization have offered insight into this distribution, but there is no quantitative *Snca* map using this technique. Furthermore, it is important to recognize that cellular RNA and protein levels may not always correlate, thereby emphasizing the importance of a comprehensive brain-wide comparative analysis encompassing both proteomic and transcriptomic dimensions (Gry et al., 2009).

We recently developed a *Snca*^{NLS} mouse allele to study the consequence of α Syn nuclear mislocalization (Geertsma et al., 2022). These mice have a nuclear localization sequence (NLS) and Flag epitope tag knocked into the C-terminus of the endogenous *Snca* gene that codes for α Syn. While characterizing these mice, we serendipitously observed that targeting the protein to

the nucleus provides unprecedented detail on the cellular and anatomical topography of this predominantly presynaptic protein. We took advantage of this cell body localization to map the regional and cellular localization of α Syn throughout the brain on the protein level and compared this to *Snca* RNA. Because the *Snca*^{NLS} mice show age-dependent phenotypes, we exclusively used young adult mice to capture α Syn localization without confounding neurodegenerative effects. We present a systematic study of its topography throughout the brain, spinal cord, retina, and enteric nervous system. We find that α Syn is broadly expressed in neurons of both the central and enteric nervous system (CNS and ENS, respectively) with specific region and neuron type enrichment. In addition, we find that α Syn is expressed in a subset of Olig2-positive cells, likely representing oligodendrocyte precursor cells. To our surprise, some cell clusters including ChAT-positive cells of the lateral reticular nucleus (LRN) and spinal cord motor neurons do not express α Syn.

Results

Whole-brain mapping reveals distinct domains of anatomical α Syn expression and distribution

To study the global topography of α Syn across the mouse brain, we used four parallel approaches, all of which use the *Snca*^{NLS} knock-in mice unless otherwise noted: 1) *Snca* RNA mapping in wild-type mice, 2) regional protein mapping with immunofluorescence and immunohistochemical staining, 3) whole brain clearing and immunolabeling, and 4) cell type marker colocalization.

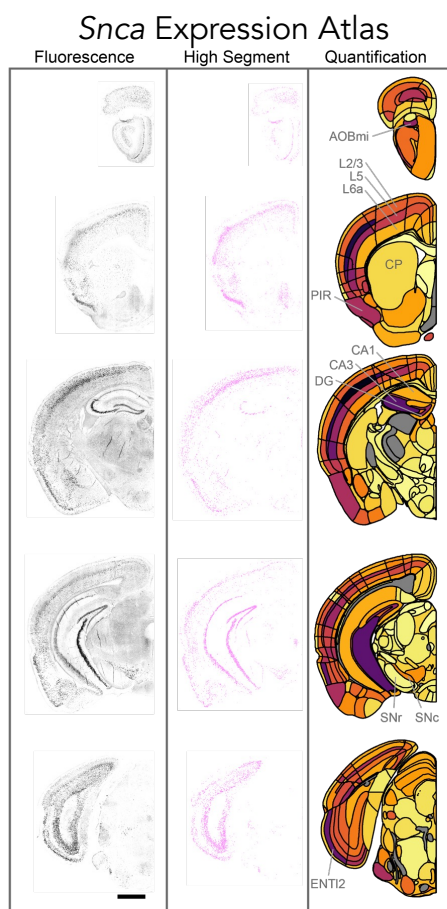
We previously found that the *Snca*^{NLS} mice have a 50% reduction in *Snca* RNA and protein, therefore we first aimed to determine if the topography of α Syn in the *Snca*^{NLS} mice is similar to *Snca* mRNA expression in wildtype mice. To determine the cellular expression of α Syn, we first stained for *Snca* mRNA in wildtype mouse brain sections (Fig. 1a). These sections were then put through a cell classifier in QuPath to classify cells as either high (greater than one standard deviation above the mean), medium (one standard deviation above or below the mean), or low (one standard deviation or more below the mean) *Snca* expression. (Supplementary Fig. 1a).

Second, we performed serial sectioning and diaminobenzidine (DAB) staining for α Syn at defined coordinates, then quantified the relative density of α Syn+ cells throughout the brain. The DAB-stained tissue from both coronal (Supplementary Fig. 2a) and sagittal (Supplementary Fig. 2b) sections were analyzed using ilastik, an image classification and segmentation tool that uses machine learning to delineate α Syn+ cells (Berg et al., 2019). Next, the α Syn+ cell density was averaged against the hematoxylin+ cell density to obtain the relative α Syn+ cell density per brain region (Supplementary Fig. 2c).

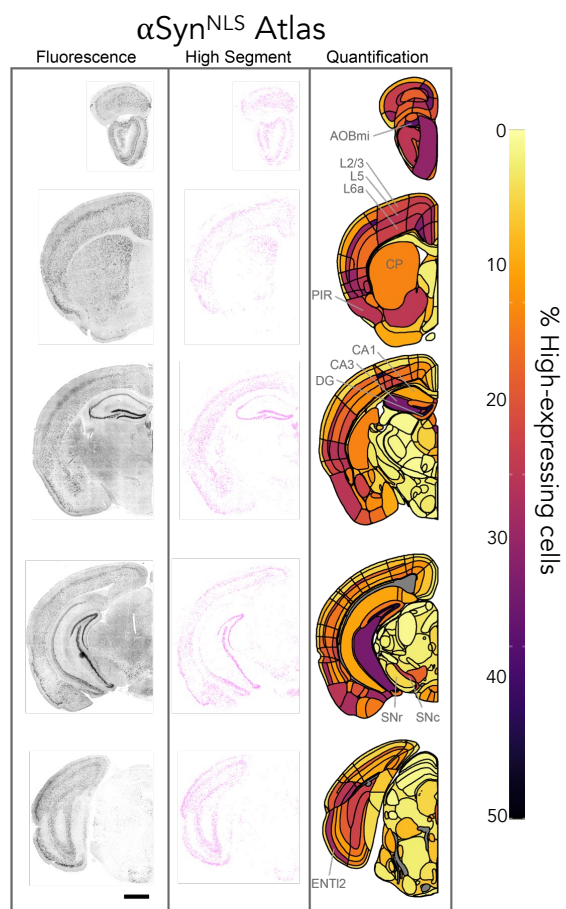
Third, to better understand the regional density of α Syn⁺ cells based on their expression levels, we generated a coronal atlas from IF-stained brain tissue at defined coordinates (Supplementary Fig. 1b). This tissue was analyzed using an adapted version of the QUINT workflow (Yates et al., 2019), which uses QuPath, QuickNII (Puchades et al., 2019), VisuAlign (NITRC: *VisuAlign - Nonlinear Adjustments after QuickNII: Tool/Resource Info*, n.d.), and Nutil (Groeneboom et al., 2020) to quantify cell classes in brains registered to the Allen Brain Atlas CCFv3 (Fig. 1b). Since the *Snc*^{NLS} model relies on functional importin proteins to harness the NLS tag and translocate α Syn into the nucleus for detection, we compared this α Syn^{NLS} atlas to the *Snc* RNA atlas. In all methods tested, we observe concordant results suggesting that *Snc* RNA broadly correlates with α Syn protein (Fig. 1c).

Fourth, we performed whole brain staining for α Syn, using the Flag epitope present in *Snc*^{NLS} mice, coupled with light sheet microscopy to generate 3D renderings of α Syn topography throughout an intact brain (Fig. 1d). Using this, we further explored the α Syn⁺ density and intensity from an intact brain, which showed similar expression patterns to our other methods (Supplementary Fig. 2d). In all methods, we found high α Syn expression throughout the cortex, especially cortical layers 4 and 5, with lower expression generally in layers 2/3 and 6. We also found high α Syn density and intensity in the hippocampus, amygdala, and olfactory bulb, including the mitral, granule, and glomerular cell layers. α Syn expression was notably low in most thalamic and mesencephalic nuclei, except for the substantia nigra pars compacta (SNc).

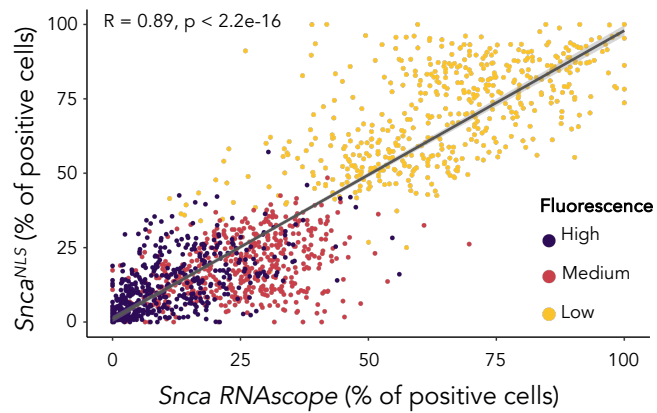
a



b



c

Comparative analysis of *Snca* RNA vs protein

d

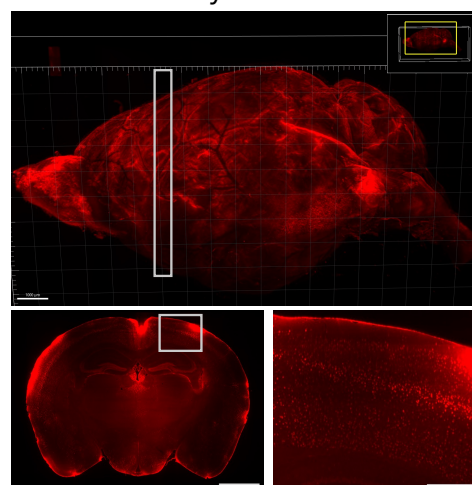
 α Syn 3D Atlas

Figure 1: A brain-wide atlas of α Syn topography. RNAScope performed on a wild-type mouse to measure *Snc*a expression (**a**) and immunofluorescent staining performed on a mouse brain measuring α Syn protein density (**b**) throughout the brain. Staining (left), segmentation (middle, high threshold), and heatmap (right) show brain region-specific expression patterns of *Snc*a or α Syn distribution, respectively. Select regions with a large percentage of high-expressing cells (AOBmi: accessory olfactory bulb, mitral layer, PIR: piriform area, BLAa: basolateral amygdalar nucleus, anterior part, CA3: field CA3 of hippocampus, DG: dentate gyrus, SNc: substantia nigra, compact part, ENTl2: entorhinal area, lateral part, layer 2) or lower percentage (CP: caudoputamen, CA1: field CA1 of hippocampus, SNr: substantia nigra, reticular part) are labeled. Cortical layers are also highlighted since these show clear differences in the percentage of high-expressing cells (L2/3: layer 2/3, L5: layer 5, L6a: layer 6a). Scale bars: 1000 μ m. **c**) A comparative analysis of (**a**) and (**b**) indicating correlation of topography between *Snc*a RNAScope in wildtype mice and α Syn immunofluorescence in *Snc*a^{NLS} mice for different intensity cut-offs. Dots represent class prevalence from individual regions. The solid line represents the line of best fit, and the shaded ribbons represent the 95% prediction intervals. The R and p values for the Pearson correlation between protein and gene expression are noted on the plots. **d**) Representative images of whole brain Flag epitope staining and imaging provide an encompassing view of α Syn topography. White boxes indicate insets. Scale bars: 1000 μ m (**d** top, **d** bottom left), 250 μ m (**d** bottom right).

Neurons express variable α Syn levels

Pathological α Syn accumulates in multiple cell types, including monoaminergic neurons, glutamatergic neurons, and oligodendrocytes (Asi et al., 2014; Braak et al., 2003; Raunio et al., 2022). One hypothesized reason for this is that susceptible cells harbour more α Syn, thus rendering them more vulnerable to the aggregation process. The most common α -synucleinopathy, PD, is characterized by aggregates within neurons; however, a rarer α -synucleinopathy, MSA, is characterized by oligodendroglial cytoplasmic inclusions (Reddy & Dieriks, 2022; Spillantini et al., 1997). We sought to characterize α Syn-expressing cell types in brain regions with high α Syn⁺ cell density, namely the olfactory bulb, motor cortex, and hippocampus (Fig. 2a). We co-stained *Snc^{ΔNLS}* mice for α Syn with cell-type markers for neurons (NeuN), astrocytes (Sox9), microglia (Iba1), and oligodendrocytes (Olig2; Fig. 2b). Congruent with previous studies, α Syn is a primarily neuronal protein, where ~80% of neurons in these brain regions consistently co-stain for α Syn, whereas only 10-20% of astrocytes, microglia, and oligodendrocytes express α Syn to a modest degree (Fig. 2c). Neurons also express significantly more α Syn than all other cell types tested. Among the other cell types, microglia and oligodendrocytes have similar intensities of α Syn between one another, both generally higher than that of astrocytes (Fig. 2d).

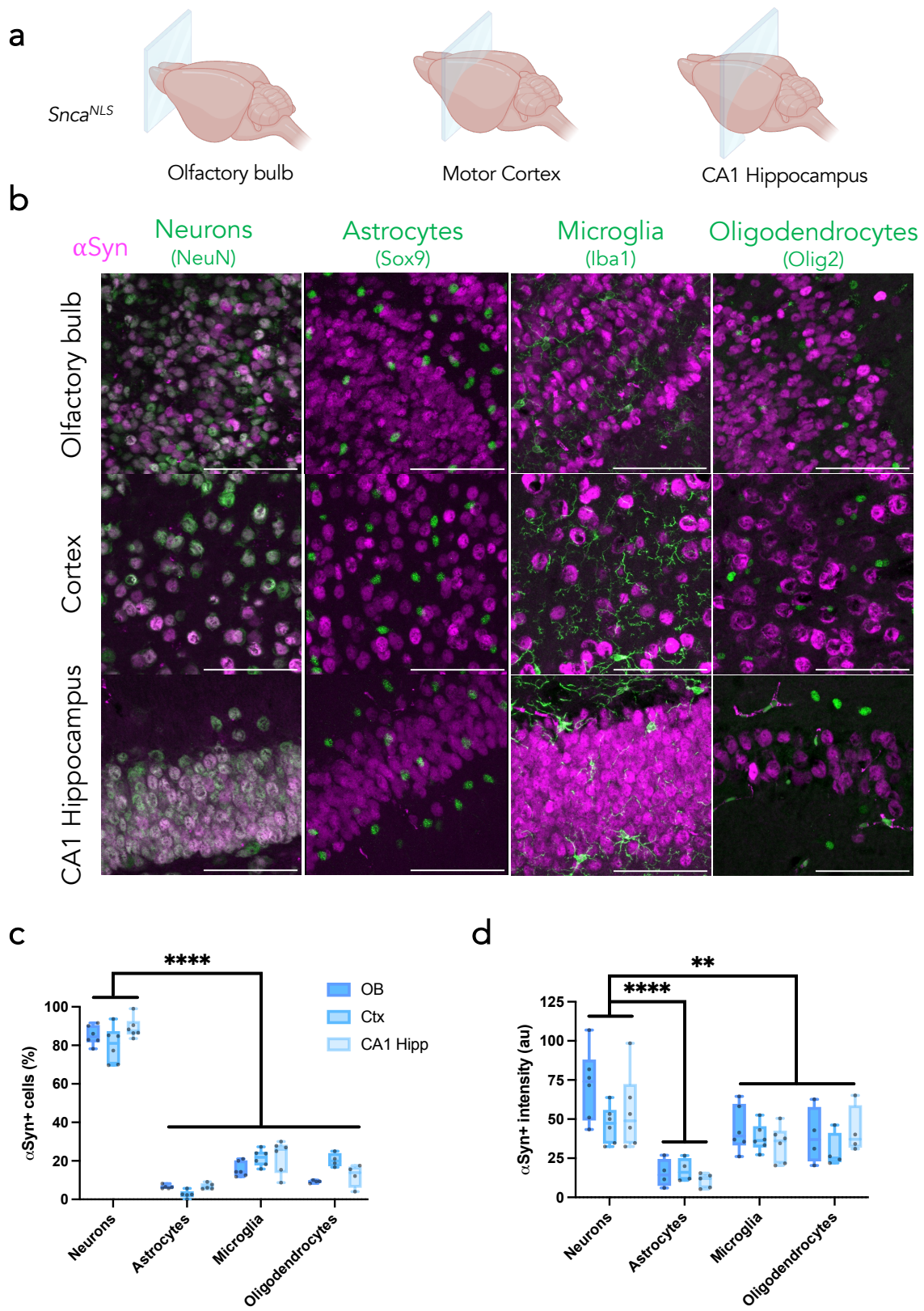


Figure 2: α Syn is predominantly expressed in neurons. **a)** Coronal planes chosen to assess different brain regions in Snca^{NLS} mice. **b)** Merged micrographs from the olfactory bulb (upper), motor cortex (middle), and CA1 hippocampus (bottom) staining for neurons (far left), astrocytes (middle left), microglia (middle right), and oligodendrocytes (right). Scale bars: 75 μ m. From these, α Syn density (**c**) and intensity (**d**) were quantified. Plotted as box plots with min to max bars. Two-way ANOVA with Tukey's post hoc, **, **** denote $p < 0.01$ and $p < 0.0001$, respectfully.

Monoaminergic cells throughout the brain broadly and consistently express α Syn whereas cholinergic neurons show variability

Since PD is characterized by selective dopaminergic neurodegeneration – and more broadly, monoaminergic susceptibility (Giguère et al., 2018) – we explored the density and intensity of α Syn protein in monoaminergic (TH+) cells of the ventral tegmental area (VTA), SNc, and locus coeruleus (LC; Fig. 3a, b). In PD, the SNc and LC have significant dopaminergic neurodegeneration, but the VTA is reported to have relatively less dopaminergic degeneration (Giguère et al., 2018). Interestingly, we found that 100% of TH+ neurons in all three brain regions co-stain for α Syn (Fig. 3b, c). Another brain region that is of importance in α Syn spreading, and PD degeneration is the dorsal motor nucleus of the vagus nerve (DMX). When staining for cholinergic neurons, we also found that 100% of ChAT+ cells of the DMX show α Syn+ staining (Fig. 3d). The DMX-adjacent nucleus ambiguous (AMB), which is not known to accumulate α Syn pathology in PD (Giguère et al., 2018), was also positive for α Syn (Fig. 3d). Intriguingly, we identified other ChAT+ cells, including the lateral reticular nucleus (LRN), that were devoid of α Syn+ staining (Fig. 3d), suggesting that α Syn expression is not part of the essential ChAT+ neuron gene expression pattern. Despite this, the LRN has been shown to harbour significant α Syn pathology in disease, which may support the prion-like spreading hypothesis of α Syn to this particular brain region in disease (Braak et al., 2003; Seidel et al., 2015). We explored other ChAT+ brain regions that are known to harbor α Syn pathology and degenerate in PD, including the medial septal nucleus (MSN; Kamalkhani & Zarei, 2022), nucleus basalis of Meynert (nbM; Lee et al., 2014), and the pedunculopontine nucleus (PPN; Henrich et al., 2020). These regions had variable levels of α Syn, however most of the cells were α Syn+, with the PPN having the brightest α Syn+ cell intensity of these three regions (Fig. 3e).

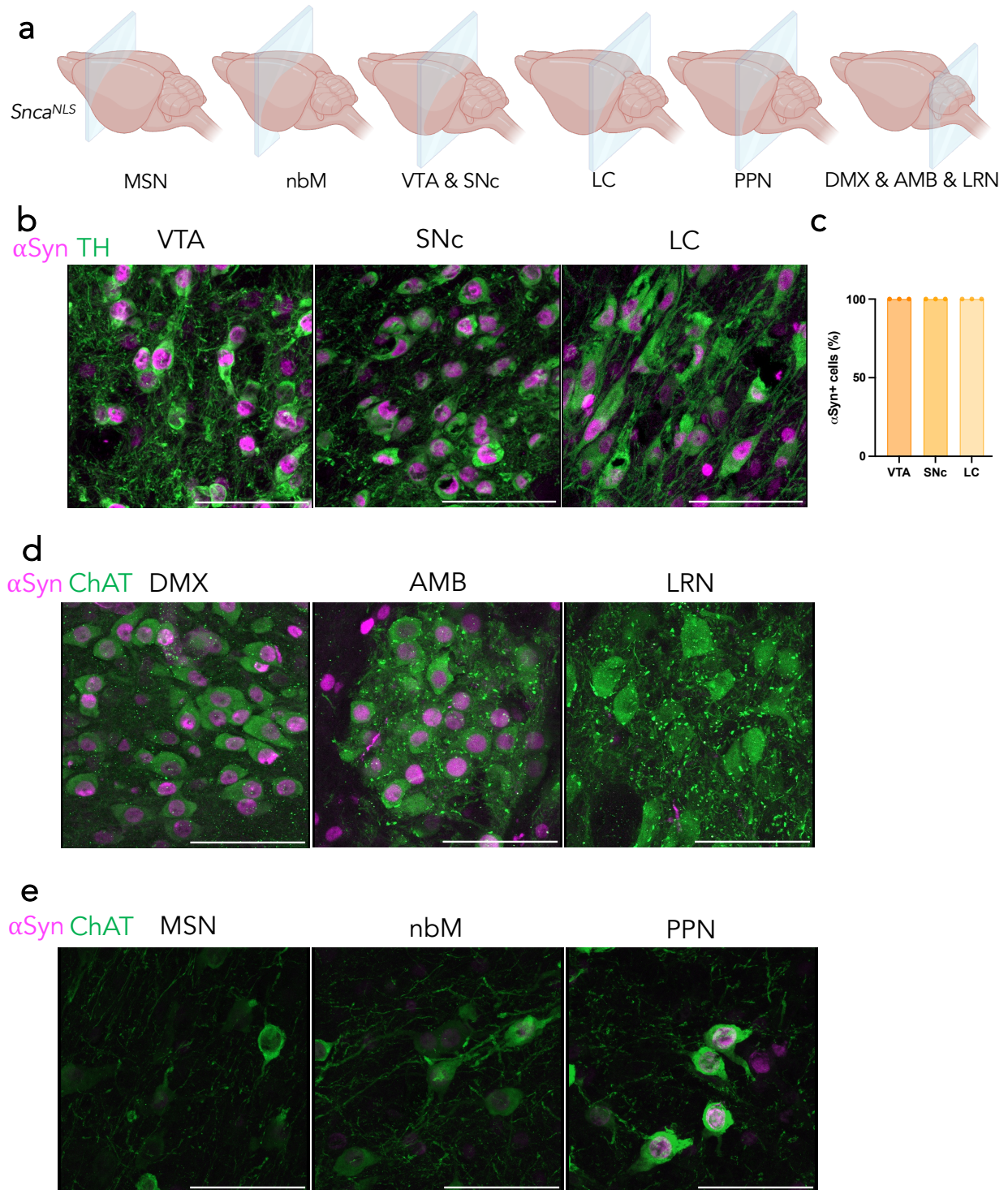


Figure 3: α Syn is highly expressed in catecholaminergic and cholinergic cell types that are vulnerable in PD. **a)** Coronal planes chosen to assess different brain regions in *Snc^α* mice. **b)** TH+ cells of the ventral tegmental area (VTA, left), substantia nigra pars compacta (SNc, middle), and locus coeruleus (LC, right) with **(c)** quantification of α Syn+ cell density. **d)** ChAT+ cells of the dorsal motor nucleus of the vagus nerve (DMX; left), nucleus ambiguus (AMB; middle), and lateral reticular nucleus (LRN; right). **e)** ChAT+ cells of the medial septal nucleus (MSN; left), nucleus basalis (nbM; middle), and pedunculopontine nucleus (PPN; right). Scale bars: 75 μ m.

Spinal cord neurons exhibit heterogeneous α Syn expression patterns

The spinal cord is not routinely studied in PD, but has gained recognition for its position as a conduit between the gut and brain and its potential role in the gut-to-brain transmission of α Syn pathology (Buhidma et al., 2023; Nardone et al., 2020; Raunio et al., 2022; Van Den Berge & Ulusoy, 2022; Vivacqua et al., 2011). Therefore, we next assessed α Syn staining patterns in the spinal cord of *Snc α ^{NLS}* mice. We analyzed sections from the cervical, thoracic and lumbar spinal cord (Fig. 4a, Supplementary Fig. 3a, 4a). Within these regions, we analyzed the dorsal horn, ventral horn, and ventral white matter. Similar to the brain, α Syn in the spinal cord is primarily neuronal; however, its localization is not homogenous (Fig. 4b, Supplementary Fig. 3b, 4b). α Syn is expressed primarily in the dorsal horn, more so in upper laminae, with α Syn⁺ cells only appearing in the ventral horn more caudally (Fig. 4c, Supplementary Fig. 3c). Additionally, α Syn intensity varies along the length of the cord, with the brightest, most concentrated α Syn⁺ cells existing in the thoracic region (Fig. 4d, Supplementary Fig. 3d). Nearly half of all NeuN⁺ cells of the thoracic and lumbar dorsal horn co-label with α Syn, a fraction significantly smaller than the neurons of the brain (>80%). Similar to the brain, however, astrocytes, microglia, and oligodendrocytes have very few cells that co-label with α Syn. Additionally, the intensity of α Syn in the dorsal horn is higher than that of the ventral horn and ventral white matter and is more consistent across cell types. These trends are all consistent in the cervical spinal cord (Supplementary Fig. 4).

To further investigate the neuronal localization of α Syn in the spinal cord, we co-stained cervical, thoracic, and lumbar sections with ChAT, a marker of motor neurons, and Pax2, a marker of GABAergic neurons. In both cases, we found that α Syn colocalizes with these markers primarily within lamina X surrounding the central canal (Supplementary Fig. 5). Since

α Syn⁺ neurons represent only 50% of the entire spinal cord neuron population (NeuN⁺; compared to >80% in the brain regions tested, Fig. 2), we turned to the harmonized atlas of single cell sequencing data to validate these findings (Russ et al., 2021; *SeqSeek*, n.d.). Consistent with our imaging, we found that *Snca* was enriched in neurons (Supplementary Fig. 6a, a', a''), specifically excitatory clusters 18 and 19 within the Sox5 family (distinguished by *Nmu* and *Tac2* expression, respectively), and inhibitory clusters 9-11 within the Pdyn family. Similarly, the harmonized atlas also illustrates the lack of overlap between *Snca* and motor neurons (Supplementary Fig. 6a''). Additionally, scRNA-Seq datasets from the mouse brain (Hu et al., 2017; Ximerakis et al., 2019) and colon (Drokhlyansky et al., 2020; *The Human and Mouse Enteric Nervous System at Single Cell Resolution - Single Cell Portal*, n.d.) show similar congruency with our atlas, illustrating the abundance of neuronal α Syn with specific sub-neuronal clusters exhibiting higher α Syn expression (Supplementary Fig. 6b-c''). These data demonstrate that α Syn exhibits a neuron subtype-specificity in the spinal cord.

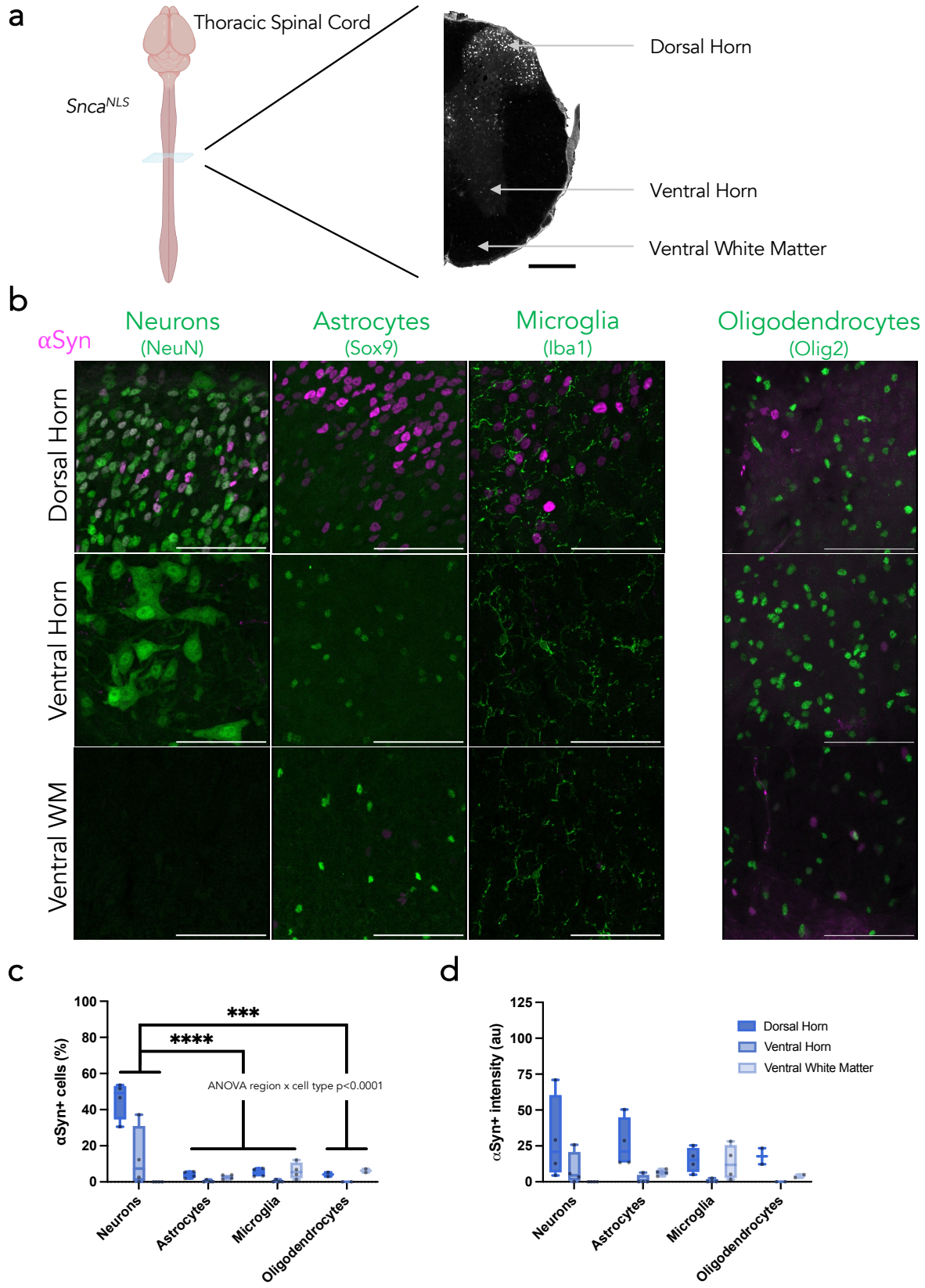


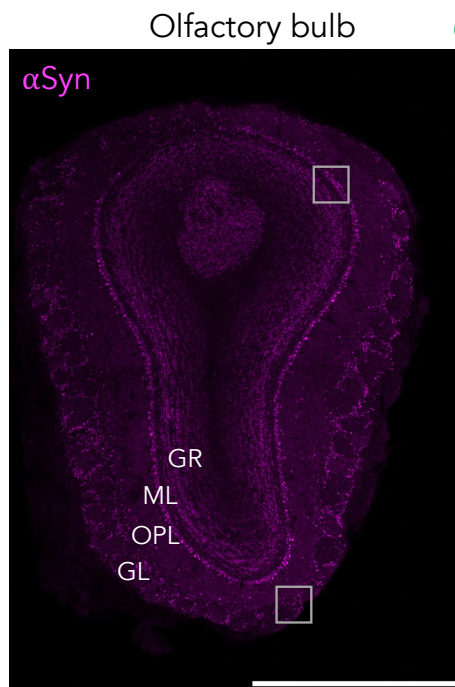
Figure 4: Diverse α Syn topography within the mouse spinal cord. **a)** Coronal plane chosen to assess different regions of the thoracic spinal cord in Snca^{NLS} mice (left) with annotations for the dorsal and ventral horns and ventral white matter (right) from a section stained for α Syn. Scale bar: 1,000 μ m. **b)** Merged micrographs from the dorsal horn (upper), ventral horn (middle), and ventral white matter (bottom) staining for neurons (left), astrocytes (middle left), microglia (middle right), and oligodendrocytes (right; different staining paradigm). Scale bars: 75 μ m. From these, α Syn density (**c**) and intensity (**d**) were quantified. Plotted as box plot with min to max bars. Two-way ANOVA with Tukey's post hoc, ***, **** denotes $p < 0.001$ and $p < 0.0001$, respectively.

Olfactory bulb and retina show distinct subpopulations of cells with high α Syn density and intensity

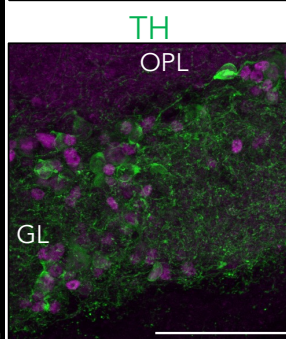
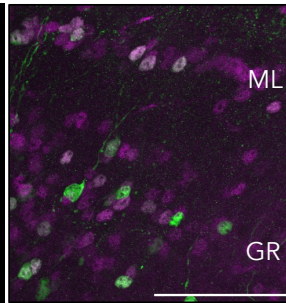
The “brain-first” model of PD suggests that α Syn pathological spread begins in the brain, typically the olfactory bulb or amygdala, before moving into the periphery (Borghammer, 2021, 2023; Horsager et al., 2020). Additional *in vivo* work has implicated the olfactory bulb as a main hub for α Syn pathology, specifically in the mitral cells (Killinger et al., 2023). We therefore investigated the olfactory bulb in more detail in *Snca^{NLS}* mice. We found prominent α Syn staining in the mitral and glomerular regions of the olfactory bulb, where we found that α Syn colocalizes with about half of all TH+ glomerular cells and calbindin/calretinin+ cells in the granular and mitral layers (Fig. 5a-c).

In conjunction with the olfactory bulb, recent studies have explored the retina as a site for α Syn pathology genesis and spread in the brain-first model. Previous studies have shown increased α Syn pathology in the retina and optic nerve, with retinal neurodegeneration in PD and other α -synucleinopathy patients (Hart de Ruyter et al., 2023; Wagner et al., 2023). These studies typically report pathology and neurodegeneration in the ganglionic cell layer and inner plexiform layer. Incidentally, these sites of pathology are where we noted most α Syn+ cells. Upon co-staining with cell-specific markers, we noted α Syn does not colocalize with ChAT+ neurons, but with calbindin/calretinin+ neurons in the ganglion and inner plexiform layers (Fig. 5d-f). We also noted higher α Syn intensity in the ganglion cell layer than in the inner plexiform layer.

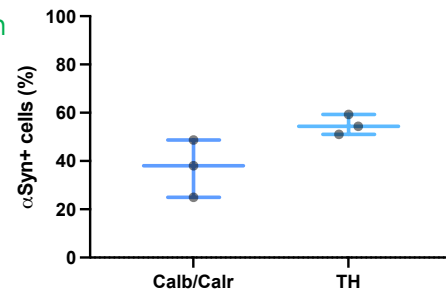
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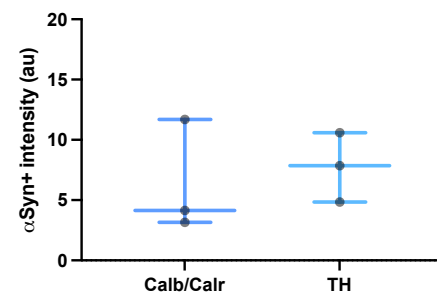
Calbindin + Calretinin



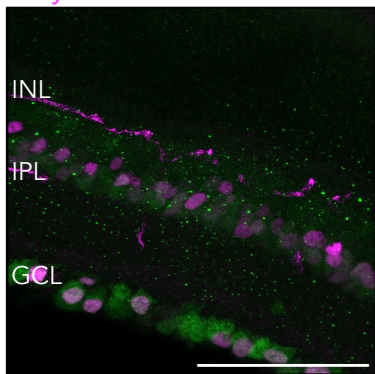
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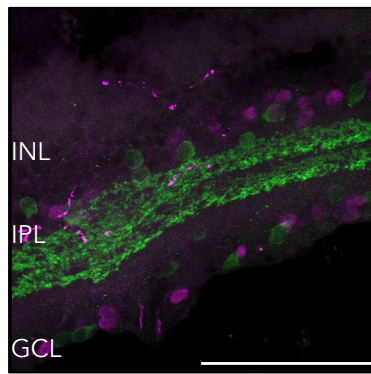
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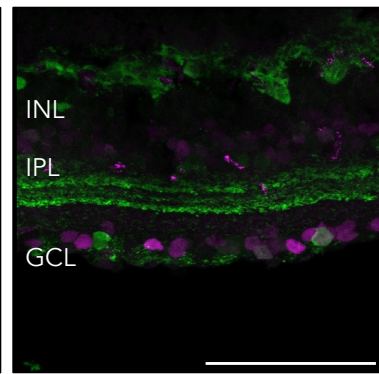
d

 α Syn NeuN

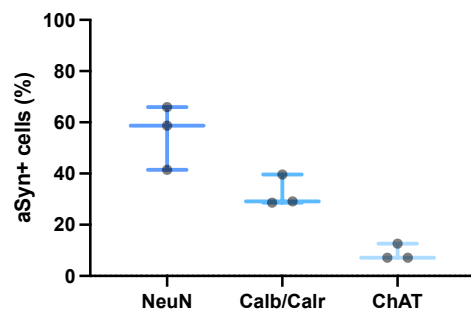
ChAT



Calbindin + Calretinin



e



f

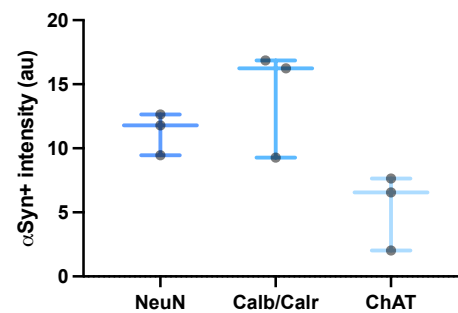


Figure 5: Diverse α Syn staining patterns within the mouse olfactory bulb and retina. a) Tiled immunofluorescent image of an olfactory bulb (left; scale bar: 1,000 μ m) shows region-specific topography of α Syn, specifically colocalizing with calbindin/calretinin in the mitral layer (upper right) and TH in the glomerular layer (lower right). Scale bars: 75 μ m. From these, α Syn density (**b**) and intensity (**c**) were quantified. Plotted as box plots with min to max bars. **d**) Cross section of Snca^{NLS} retina co-stained for NeuN (left) ChAT (middle) or calbindin/calretinin (right). From these, α Syn density (**e**) and intensity (**f**) were quantified. Plotted as box plots with min to max bars. Glomerular layer (GL), outer plexiform layer (OPL), mitral layer (ML), granule layer (GR), ganglionic cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL).

All enteric neurons of the stomach and duodenum have α Syn protein of varying intensity

Gastrointestinal symptoms, including constipation, are known prodromal symptoms of PD and α Syn pathology has been observed in the gut tissue of PD patients (Beach et al., 2016; Braak et al., 2006; Corbillé et al., 2016). We therefore aimed to characterize the cells that may be contributing to this pathological burden. Since we previously noted that α Syn primarily resides in neurons, we focused on the enteric neurons. We performed whole-mount staining of the stomach and duodenum of *Snc α ^{NLS}* mice then co-stained for α Syn and Tuj1/HuC/D to mark enteric neurons (Fig. 6a). This whole-mount preparation preserves both enteric neurons and longitudinal muscle. Here, we find α Syn exclusively within enteric neurons of both the stomach and duodenum with considerable variability in the relative amount of α Syn between neurons (Fig. 6b). This finding is consistent across the stomach and duodenum and is in line with recent enteric nervous system single-cell sequencing findings (Supplementary Fig. 6c, c', c''; Morarach et al., 2021; *The Human and Mouse Enteric Nervous System at Single Cell Resolution - Single Cell Portal*, n.d.).

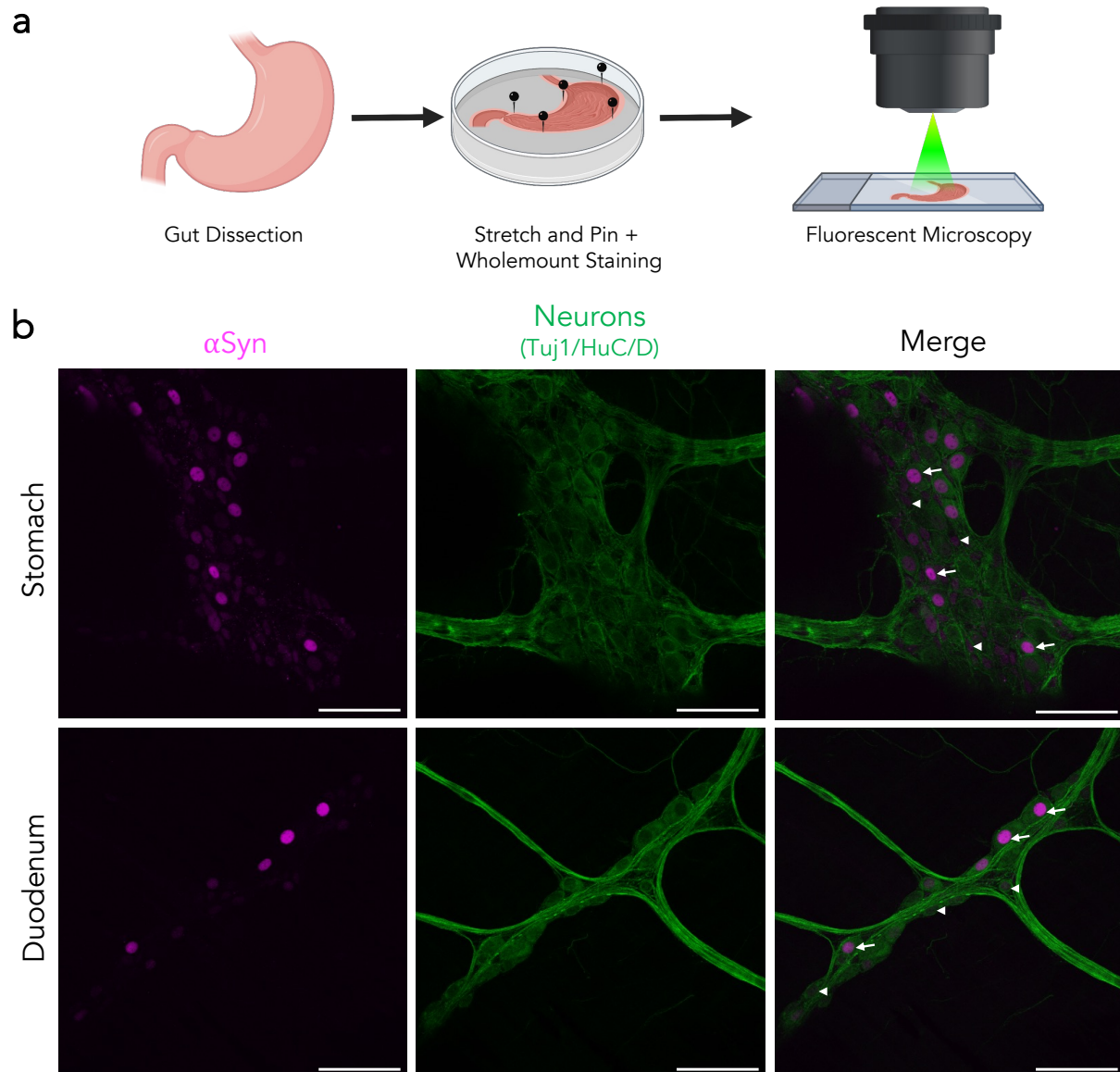


Figure 6: Varying intensities of α Syn expression throughout the gut. **a)** $Snc\alpha^{NLS}$ gut tissue was flushed and stained whole-mount prior to immunofluorescent staining and imaging. **b)** α Syn shows 100% colocalization with neuronal markers in the stomach (upper) and duodenum (lower). Arrowheads denote low α Syn, arrows denote high α Syn intensity. Scale bars: 75 μ m.

Discussion

In this study, we used our *Snc α ^{NLS}* mouse model to characterize the topography of α Syn systematically throughout the brain and in other PD-relevant tissues. We found that α Syn is expressed predominantly in neurons and that, while it is expressed in some of the non-neuronal cells tested, it is at much lower levels. To our surprise, cholinergic neurons express α Syn to varying degrees, with some expressing high levels of α Syn (e.g. DMX) while others are virtually devoid of α Syn (e.g. motor neurons of the spinal cord). It was interesting to note that olfactory neurons (particularly TH⁺, periglomerular cells) were highly enriched for α Syn whereas retinal ganglion cells and amacrine cells expressed very low levels of α Syn, which bordered on the lower limit of detection. This is consistent with recent findings that suggest the olfactory bulb is likely a richer source of seed-competent α Syn than the retina however contrary to a 2016 study that found wildtype α Syn does not colocalize with TH⁺ cells in the periglomerular olfactory bulb (Pérez-Acuña et al., 2023; Rey et al., 2013, 2016, 2018; Taguchi et al., 2016). This discrepancy may be due to the presynaptic/cytosolic localization of α Syn in wildtype mice whereby TH⁺ neurons in the periglomerular region of the olfactory bulb can project outside this layer, making interpretation of wildtype α Syn localization difficult. While our findings in mice are largely consistent with what is known of α Syn topography in the human brain, we acknowledge that these organisms may exhibit different patterns of α Syn localization. However, the patterns of α Syn expression we have identified may inform our understanding of human neuropathology. For example, the amygdala and substantia nigra pars compacta, which are heavily impacted by Lewy pathology in PD and other diseases, show high α Syn expression. In contrast, another region with high α Syn expression, the CA3 region of the hippocampus, is

generally less impacted by Lewy pathology, suggesting that cellular resilience factors and neuronal connectivity also play a role in disease. However, when the hippocampus is impacted in patients, Lewy pathology is primarily in CA2/3, not CA1, consistent with higher expression of α Syn relating to higher likelihood of developing Lewy pathology (Adamowicz et al., 2017; Coughlin et al., 2020; Dickson et al., 1991).

Astrocytes, microglia, and oligodendrocytes all expressed α Syn but at very low levels. Some populations of Olig2⁺ cells, however, expressed a considerable amount of α Syn. These are likely oligodendrocyte precursor cells (OPCs) which have previously been reported to express a relatively high level of α Syn (Cahoy et al., 2008; Asi et al., 2014; Djelloul et al., 2015; Kaji et al., 2018; Zhu et al., 2022; Azevedo et al., 2022). The role that α Syn plays within these cells remains enigmatic, but higher native expression in OPCs, in concert with genetic and/or environmental insults, may predispose a cell to future pathology in diseases like MSA, where α Syn pathology principally occurs in oligodendrocytes.

Most α Syn⁺ cells of the spinal cord lie within the upper laminae of the dorsal horn, with very few α Syn⁺ cells in the ventral horn and ventral white matter. Concordantly, α Syn⁺ aggregates have been found in the dorsal horn of PD spinal cord tissue (Del Tredici & Braak, 2012; Sumikura et al., 2015). The upper laminae of the dorsal horn are largely comprised of the substantia gelatinosa (primarily GABAergic neurons) and the nucleus proprius (primarily somatostatin, calretinin, and VGLUT3⁺ neurons; Lai et al., 2016). These regions receive inputs from many higher order brain structures, including the locus coeruleus (Sheikh & Dua, 2023). It has also been suggested that α Syn pathology spreads from the dorsal horn of the spinal cord to the dorsal root ganglia via the dorsal root (Sumikura et al., 2015). However, additional work

needs to be done to validate the specific cell types responsible for spreading, retaining α Syn pathology, and degenerating in disease.

Myenteric neurons of the enteric nervous system express a considerable amount of α Syn. Interestingly, this level is highly varied between neuronal subtypes. This finding is consistent with a recent single cell transcriptomic dataset that highlights an enrichment of *Snca* in enteric neuron classes (ENCs) 5, 9, 10 and 11 (Morarach et al., 2021). Interestingly, ENC11 is defined by a high expression of TH and Calb2, markers that cluster with high α Syn expressing cells like dopamine neurons. This expression pattern could imply that it is these dopaminergic neurons of the gut that harbour more α Syn protein, and may be more vulnerable in disease, however additional research is required on the implications of α Syn protein load in the gut and how this translates to pathological burden in disease.

The development of this topographical atlas of α Syn expression sets the stage for future studies exploring the impact of neuronal α Syn on subsequent vulnerability to developing Lewy pathology. First, this atlas provides a newfound level of insight in healthy, young animals. Future work, looking at the impact of PD-relevant risk factors such as age, toxin or genetic exposure, or viral insults will shed important light on modes of native α Syn regulation. Second, it remains unclear whether the native abundance of α Syn affects the susceptibility of a cell to neurodegeneration. Seeding-based experiments suggest that cells with higher α Syn are more vulnerable to pathology (Henderson et al., 2019; Luna et al., 2018), while human imaging studies have suggested that regional atrophy in PD is related to anatomical connectivity (Dagher & Zeighami, 2018). Cellular milieu likely also plays a role in α Syn vulnerability, with studies finding different α Syn fibril strains forming in different cell types, leading to differing levels of pathogenicity (Just et al., 2022). It is likely that these factors (anatomical connectivity, gene

expression, and cell environment) work in concert to make certain neurons and brain regions vulnerable to developing Lewy pathology (Henderson et al., 2022). Future work looking to selectively target α Syn-rich versus α Syn-poor circuits will provide much-needed understanding of the role of endogenous α Syn dosage and cellular vulnerability. Lastly, given the increased focus on α Syn-lowering therapeutics (e.g. immunotherapy, antisense oligonucleotides, aggregation inhibitors, viral approaches), this atlas will provide insight into why certain cell types may be more resistant to these translational modalities, providing ways to better target therapeutics and circumvent associated pitfalls.

Materials and Methods

Mouse Husbandry

All mice were housed with up to 5 mice per cage on a 12-hour light-dark cycle. Mice were fed *ad libitum* and all husbandry was performed by the uOttawa Animal Care and Veterinary Services staff. All animal work was performed under the breeding protocols (CMMb-3654 and CMMb-3904) approved by the uOttawa Animal Care Committee. The *Snc^α^{NLS}* mouse line used in this study is available through the Jackson Laboratory (036763; RRID:IMSR_Jax:036763). All housing and procedures at Van Andel Institute were performed according to the NIH Guide for the Care and Use of Experimental Animals and approved by the Van Andel Institute Institutional Animal Care and Use Committee (IACUC). C57BL/6J mice were purchased from the Jackson Laboratory (000664; RRID:IMSR_JAX:000664). Both male and female mice were used in this study and all mice were between 9-13 weeks old.

Tissue collection

Mice were sedated with an intraperitoneal injection of 120 mg/kg Euthanyl (DIN00141704). Next, they were transcardially perfused with 10 mL 1X phosphate buffered saline (PBS) + 10 U/mL heparin (Millipore Sigma, H3393-50KU) followed by 10 mL 4 % paraformaldehyde (PFA). Brains were extracted and incubated in 4 % PFA overnight at 4 °C with gentle rocking. The brains were then washed twice with 1X PBS ([dx.doi.org/10.17504/protocols.io.b5swq6fe](https://doi.org/10.17504/protocols.io.b5swq6fe)). Brains used for Fig. 1a-c, and Supplementary Fig. 1 were processed into paraffin via sequential dehydration and perfusion with paraffin under vacuum (70 % ethanol for 1 hour, 80 % ethanol for 1 hour, 2 times 95 % ethanol for 1 hour, 3 times 100 % ethanol for 1 hour, 2 times xylene for 30 minutes, paraffin for 30 minutes at 60 °C, paraffin for 45 minutes at 60 °C). Brains were then embedded in paraffin blocks, cut into 6 µm sections and mounted on glass slides. Brains and spinal cords used for Fig. 2-5, and Supplementary Fig. 2-5 were dehydrated in serial sucrose solutions for 48 hours each in 10 %, 20 %, then 30 % sucrose. Next, the organs were flash frozen in ~ -40 °C isopentane then cryosectioned at 20 µm and stored as free-floating tissue in 1X PBS with 0.02 % sodium azide. Subsequent steps are protocol-dependent and outlined in downstream methods. See Supplementary Table 1 for a comprehensive list of all antibodies/kits used in this study.

Snca Expression, α Syn^{NLS}, and α Syn DAB Atlas

In situ hybridization

In situ hybridization was performed with RNAscope® Multiplex Fluorescent Reagent Kit v2 (ACD, Cat #323270) using recommended conditions and supplied reagents. Paraffin-embedded

tissue was freshly sectioned and dried. When it was not going to be used immediately, slides were vacuum-sealed and stored at 4 °C. Slides were baked in a dry oven for 1 hour at 60 °C and used within one week. Slides were de-paraffinized with 2 sequential 5-minute washes in xylenes, followed by 2 washes in 100 % ethanol for 2 minutes. Slides were then dried for 5 minutes at 60 °C. Slides were treated with hydrogen peroxide for 10 minutes at room temperature and washed two times with distilled water. Target retrieval was performed in target retrieval reagents in the BioGenex EZ-Retriever System for 15 minutes at 99 °C. Slides were then washed with distilled water for 15 seconds and transferred to 100 % ethanol for 3 minutes before being dried for 5 minutes at 60 °C.

Slides were incubated in protease plus in a humidified tray in a hybridization oven (Boeckel Scientific 240200) for 30 minutes at 40 °C. Slides were washed 2 times with distilled water. RNAscope® probes were added to slides and incubated for 2 hours at 40 °C. The following probe was used: *Snca* (RNAscope probe, Mm-Snca-C1, 313281). Slides were washed twice for 2 minutes with wash buffer and incubated in Amp 1 for 30 minutes at 40 °C. The wash and Amp incubation was repeated for Amp 2 and Amp 3, except Amp 3 was only incubated for 15 minutes. Slides were washed twice for 2 minutes with wash buffer and incubated in HRP-C1 for 15 minutes at 40 °C. Slides were washed twice for 2 minutes with wash buffer and incubated in Opal 520 (Perkin Elmer FP1487A) for 30 minutes at 40 °C, washed twice for 2 minutes with wash buffer, incubated in HRP blocker for 15 minutes at 40 °C, and washed twice for 2 minutes with wash buffer. Slides were mounted with coverglass in ProLong gold with DAPI (Invitrogen, Cat#P36931) and imaged at 20x magnification on a Zeiss AxioScan 7 microscope.

Immunofluorescence

Slides were de-paraffinized with 2 sequential 5-minute washes in xylenes, followed by 1-minute washes in a descending series of ethanols: 100 %, 100 %, 95 %, 80 %, 70 %. Slides were then incubated in deionized water for one minute prior and transferred to the BioGenex EZ-Retriever System where they were incubated in antigen unmasking solution (Vector Laboratories; Cat# H-3300) and microwaved for 15 minutes at 95 °C. Slides were allowed to cool for 20 minutes at room temperature and washed in running tap water for 10 minutes. Slides were washed for 5 minutes in 0.1 M Tris, then blocked in 0.1 M Tris/2 % fetal bovine serum (FBS) for 2 hours. Slides were incubated in primary antibody in 0.1 M Tris/2 % FBS in a humidified chamber overnight at 4 °C. Primary antibodies were rinsed off with 0.1 M tris and incubated in 0.1 M Tris/2 % FBS for 5 minutes. Slides were then incubated in the dark for 3 hours at room temperature with secondary antibodies in 0.1 M Tris/2 % FBS. Slides were rinsed three times for 10 minutes in 0.1 M Tris in the dark, then mounted with coverglass in ProLong gold with DAPI (Invitrogen, Cat# P36931). Fluorescent slides were imaged at 20x magnification on a Zeiss AxioScan 7 microscope.

Cell detection and classification

Our study included 99 sections from 4 *Snca*^{NLS} mice for immunofluorescence and 49 sections from 4 C57Bl/6J mice for *in situ* hybridization. Stained slides scanned on a Zeiss AxioScan 7 at 20x magnification were imported into QuPath (version 0.2.3, RRID:SCR_018257) or newer for analysis. Individual cells were identified using the Cell Detection feature that allows detection

based on DAPI stain intensity. Cell detection parameters such as background radius, sigma, and threshold were adjusted to optimize cell detection across all brain sections.

After cell detection, cell classification was completed in QuPath using the object classification feature. QuPath was trained on a subset of annotations to distinguish between different cell types based on signal intensity. To train the classifier, cells were detected by DAPI then classified according to the nuclear expression intensity of α Syn staining or whole-cell intensity of *Snca* staining. Thresholds for the intensity classifications were calculated by averaging the mean intensity of all cells. Three classes were then generated: 1) Low: one standard deviation or less below the mean cellular intensity; 2) Medium: one standard deviation below or above the mean cellular intensity; 3) High: one standard deviation or more above the mean cellular intensity. This classifier was then applied to all brain sections. Each class was assigned a different colour and exported as .png files for subsequent analysis.

Mouse Brain Registration

Images were registered to the Allen Brain Atlas CCFv3 using a modified version of the QUINT workflow²¹. An RGB image of each section was exported from QuPath as a .png file, downsampled by a factor of 12, to use for spatial registration in QuickNII (RRID:SCR_016854)²². A segmentation image was created by exporting a colour-coded image of classified cells by category on a white background to use as the segmentation input in Nutil (RRID:SCR_017183). Brain images were uploaded in Filebuilder and saved as an XML file to be compatible with QuickNII. Following the spatial registration of the mouse brain sections to the Allen Mouse Brain Atlas CCFv3 in QuickNII, a JSON file was saved for use in (VisuAlign, RRID:SCR_017978). Brain sections were imported into VisuAlign to perform non-linear warp

transformations and align all brain regions. Anchor points were generated in the atlas overlay and moved to the corresponding location on the brain section. Final alignments were exported as .flat and .png files for use in Nutil⁷⁸.

Nutil was used for the quantification and spatial analysis of the identified cell types in specific regions of the mouse brain. Individual classes were identified for quantification via their HTML colour code assigned in QuPath. Nutil generated object counts from each individual classification within each region of the Allen Mouse Brain Atlas using the registration from QuickNII and VisuAlign. The percentage of each class was calculated for each region and used for subsequent plotting and analysis. Anatomical heatmaps were generated using custom R code (https://github.com/vari-bbc/Mouse_Brain_Heatmap). Raw output values and R code are available at <https://zenodo.org/records/10685351>.

DAB staining: [dx.doi.org/10.17504/protocols.io.b5s9q6h6](https://doi.org/10.17504/protocols.io.b5s9q6h6)

Cryosectioned tissue were incubated in 0.9 % hydrogen peroxide (Millipore Sigma, 216763-500ML) for 10 minutes at room temperature. The following diaminobenzidine staining was performed using the Vectastain Elite ABC HRP Mouse Kit (Vector Laboratories, PK-6102). Briefly, tissue was incubated in blocking buffer (1.5 % triton X-100 + 5 % Vectastain Horse Serum in 1X PBS) for 1 hour at room temperature following by incubation in primary antibody solution overnight at 4 °C. Next, tissue was incubated in secondary antibody diluted in blocking buffer for 1 hour, then ‘A+B’ solution (mixed 30 minutes prior to use) for 1 hour at room temperature. Tissue was then exposed to diaminobenzidine for 2 minutes using the DAB Peroxidase HRP Substrate Kit (Vector Laboratories, SK-4100) then washed with 1X PBS. Tissue was dehydrated in 50 %, 70 %, 95 %, then 100 % ethanol for 1 minute each, then 100 % xylenes

for 5 minutes. Finally, tissue was coverslipped with Permount mounting medium (Fisher Scientific, SP15-100) and #1.5 coverslips. Stained slides were imaged at 20x magnification using an Axio Scan Z1 Slide Scanner and exported as .czi files for subsequent analysis.

Hematoxylin staining

Cryosectioned tissue was stained using the H&E Staining Kit (Abcam, ab245880) following kit instructions. Briefly, free-floating sections were incubated in hematoxylin for 5 minutes then washed with 2 changes of water, blueing reagent for 15 seconds, then washed in water again. Tissue was then mounted on a superfrost plus slide and dehydrated in 50 %, 70 %, 95 %, then 100 % ethanol for 1 minute each then 100 % xylenes for 5 minutes before coverslipping with Permount mounting medium (Fisher Scientific, SP15-100) and #1.5 coverslips. Stained slides were imaged at 20x magnification using the Axio Scan Z1 Slide Scanner and exported as .czi files for subsequent analysis.

α Syn DAB Atlas quantification

All tissue slices were annotated to specific Allen Brain Atlas coordinates then converted to .tiff files using Fiji ImageJ (version 2.3.0/1.53q, RRID:SCR_002285). The ‘pixel classification’ pipeline from ilastik software (version 1.3.2, RRID:SCR_015246) was used for to delineate positive cells and three images were used in training the machine learning software. Segmented images were opened in Fiji and brain regions were manually outlined and added to an ROI manager. The ‘analyze particles’ function was used to count positive cells greater than 5 pixels in size, corresponding to 1 positive cell. The number of α Syn⁺ cells was divided by the number of hematoxylin positive cells and multiplied by 100 to give then relative α Syn⁺ cell density.

Whole brain clearing and imaging

Whole *Snca*^{NLS} mouse brains were processed using the SHIELD protocol by LifeCanvas Technologies (Park et al., 2019). Samples were cleared for 7 days with Clear+ delipidation buffer then immunolabeled using SmartLabel. Each sample was labelled with 5 µg mouse anti-NeuN (RRID:AB_2572267) and 4 µg goat anti-Flag (RRID:AB_299216) followed by fluorescent secondary antibodies. Samples were incubated in EasyIndex for a refractive index of 1.52 and imaged at 3.6x using SmartSPIM microscopy. Images were tile-corrected, de-striped, and registered to the Allen Brain Atlas (<https://portal.brain-mapping.org>). The NeuN channel was registered to the 8-20 atlas-aligned reference samples using successive rigid, affine, and b-spline warping (SimpleElastix: <https://simpleelastix.github.io>). Average alignment to the atlas was generated across all intermediate reference sample alignments to serve as the final atlas alignment value per sample. Fluorescent measurements from the acquired images were projected onto the Allen Brain Atlas to quantify the total fluorescence intensity per region defined by the Allen Brain Atlas. These values were then divided by the volume of the corresponding regional volume to calculate the intensity per voxel measurements. Raw output files from LifeCanvas are available at <https://zenodo.org/records/10685351>. Data was plotted using Prism (version 9.5.1, RRID:SCR_002798) as a Box plot with min to max bars.

Cell-specific staining and quantification

Organ isolation: [dx.doi.org/10.17504/protocols.io.b5swq6fe](https://doi.org/10.17504/protocols.io.b5swq6fe)

Following intracardial perfusion (see tissue collection method above), brains, spinal cords, and retina from *Snca*^{NLS} mice were extracted and incubated in 4 % PFA for 72 hours at 4 °C with gentle rocking. Brain and spinal cords were next dehydrated in a 3-step sucrose sequence with

10, 20, and 30 % sucrose for 24 hours each. Finally, brains and spinal cord were flash frozen for 1 minute at -40 °C isopentane, suspended in OCT, then cryosectioned at 40 µm. Eyes were incubated in 70 % ethanol following fixation, then retinas were isolated, suspended in OCT, and cryosectioned at 20 µm.

Immunofluorescent staining: [dx.doi.org/10.17504/protocols.io.b5s5q6g6](https://doi.org/10.17504/protocols.io.b5s5q6g6)

Cryosectioned, free-floating tissue sections were incubated in blocking buffer (0.5 % Triton X-100 (Sigma-Aldrich, T8787-100ML) + 10 % normal horse serum (Sigma-Aldrich, H0146-5ML) in 1X PBS) for 1 hour at room temperature. Next, sections were incubated in 1° antibody overnight at 4 °C then fluorescently conjugated 2° antibody for 1 hour at room temperature. Free-floating sections were mounted on SuperFrost+ slides then coverslipped with fluorescent mounting medium (Agilent, S302380-2) and #1.5 coverslips.

ChAT staining: [dx.doi.org/10.17504/protocols.io.rm7vzxbz4gx1/v1](https://doi.org/10.17504/protocols.io.rm7vzxbz4gx1/v1)

Cryosectioned tissue were incubated in blocking buffer (0.1 % Triton X-100 (Sigma-Aldrich, T8787-100ML) + 10 % normal horse serum (Sigma-Aldrich, H0146-5ML) + 0.5 % gelatin (Sigma-Aldrich, G1393-100ML) in 1X PBS) for 2 hours at room temperature. Next, sections were incubated in 1° antibody for 48 hours at 4°C then fluorescently conjugated 2° antibody for 2 hours at room temperature. Free floating sections were mounted on SuperFrost+ slides then coverslipped with fluorescent mounting medium (Agilent, S302380-2) and #1.5 coverslips.

Brain quantification

Stained sections were imaged with a Zeiss LSM800 AxioObserver Z1 microscope at 20x with Z-stack. Images were then 3D projected and all channels were merged. Each α Syn⁺ cell nucleus that co-labels with the specific cell marker was circled and the intensity was measured in Fiji ImageJ (version 2.3.0/1.53q) with the ROI manager. The background signal was measured by circling 3 separate regions of α Syn⁻ space and measuring the intensity with the ROI manager. To quantify, the background values were averaged and subtracted from each cell-specific intensity measurement. These intensity values were plotted in a nested graph using Prism (version 9.5.1, RRID:SCR_002798) as a Box plot with min to max bars and analyzed using two-way ANOVA with Tukey's post hoc analysis.

ENS immunofluorescence: [dx.doi.org/10.17504/protocols.io.14egn3wxpl5d/v1](https://doi.org/10.17504/protocols.io.14egn3wxpl5d/v1)

Following euthanasia and perfusion of animals, stomach and duodenum were collected, washed, stretched, pinned flat on Sylgard-coated Petri dishes, and fixed overnight at 4°C in phosphate buffer saline solution (PBS) containing 4% (vol/vol) paraformaldehyde (Sigma Alsich cat# P6148). Layers of tissue containing the myenteric plexus were separated by microdissection. Tissues were permeabilized at room temperature (RT) for 2 hours in a 10 % fetal bovine serum (FBS)/PBS blocking buffer containing 0.5% Triton X-100 (Sigma Aldrich, cat# X100), and incubated overnight at 4°C with the following primary antibodies diluted in the blocking buffer: rabbit monoclonal anti- α -synuclein. Following incubation with primary antibodies, tissues were washed with 1X PBS and incubated for 1 hour at room temperature in secondary antibodies. Tissues were then washed with 1X PBS and mounted with ProLong Gold antifade reagent (Thermo Fisher Scientific, cat# P36931). Confocal images were acquired using a Nikon

A1plus-RSi confocal microscopy under a 40x objective and were analyzed with Image J software.

Statistical analysis

All statistical analyses were performed using GraphPad Prism (version 9.5.1) unless otherwise specified using a Two-way ANOVA with Tukey's post hoc or Unpaired t-test, specified in the methods above. All statistical analyses with all raw values that were used to generate each figure at <https://zenodo.org/records/10685351>.

Data availability:

The datasets generated and/or analyzed during the current study (including Supplementary Tables 2 and 3) are available on Zenodo at <https://zenodo.org/records/10685351>

Code availability:

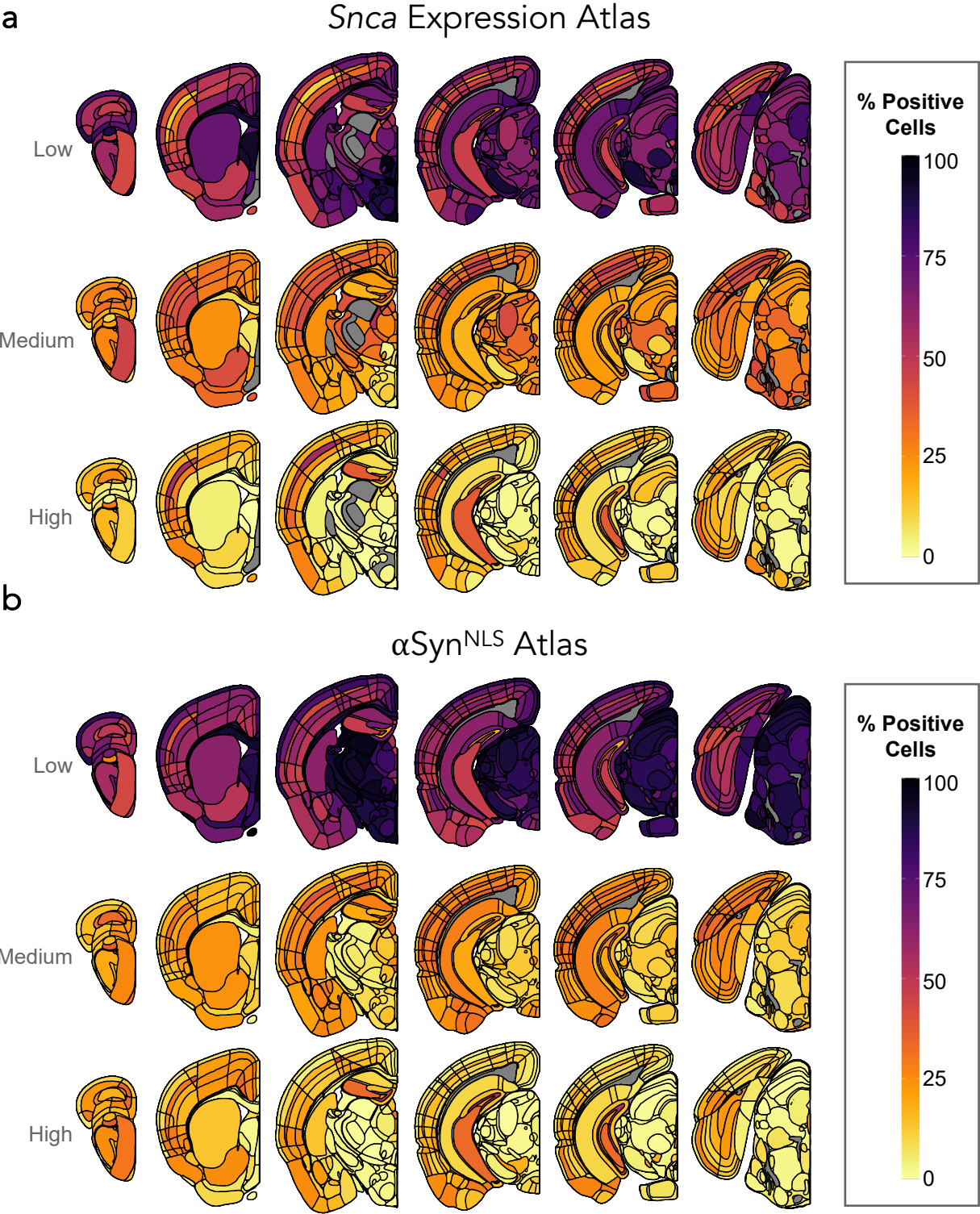
The underlying code for Fig. 1c is available with the raw data that went into that analysis at <https://zenodo.org/records/10685351>.

Acknowledgements:

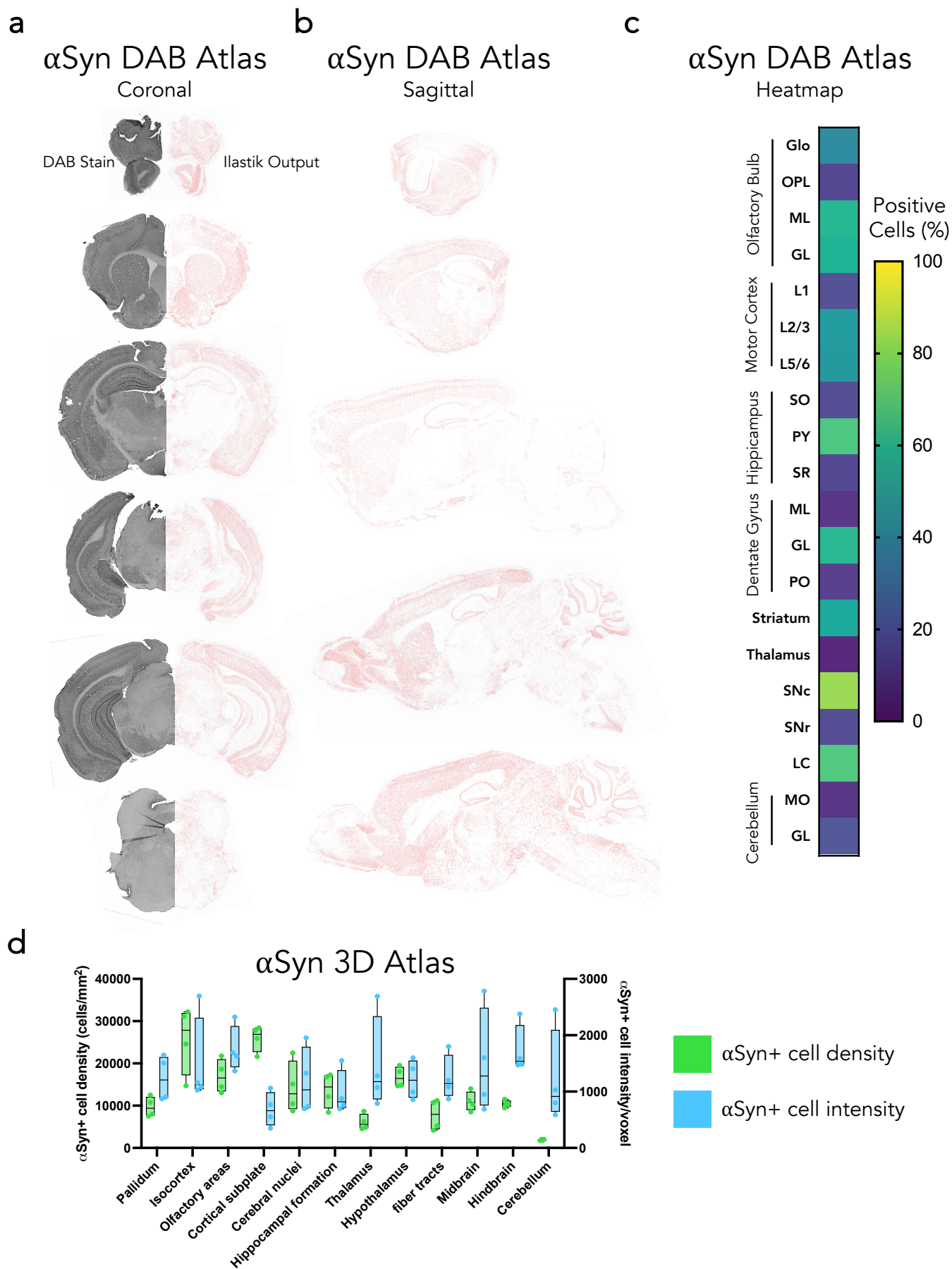
The authors thank Michelle Seguin and Meghan Heer for valuable insight into the early phases of this project and assistance with colony maintenance. This research was funded in part by Canadian Institutes of Health Research (PJT-169097, to M.W.C.R.); the Parkinson Canada New Investigator Award (2018-00016, to M.W.C.R.); Aligning Science Across Parkinson's [ASAP-020625 to M.W.C.R., ASAP-020616 to M.X.H.] through the Michael J. Fox Foundation for

Parkinson's Research (MJFF); Canadian Institutes of Health Research Canada Graduate Scholarship-D (FBD-181526, to H.M.G.); Dave and Jill Hogg and Toth Family Parkinson Research Consortium Fellowships (Z.A.F.). For the purpose of open access, the author has applied a CC BY public copyright license to all Author Accepted Manuscripts arising from this submission. The authors thank the following Core facilities from the University of Ottawa for use of their facility, equipment, and expertise: Cell Biology and Imaging Acquisition Core (RRID: SCR_021845) and Louise Pelletier Histology Core (RRID: SCR_021737). Fig. 2a, 3a, 4a, 5b, 6a, Supplementary Fig. 3a, and 4a were generated, in part, using Biorender.com. All histograms were generated with Prism 9.

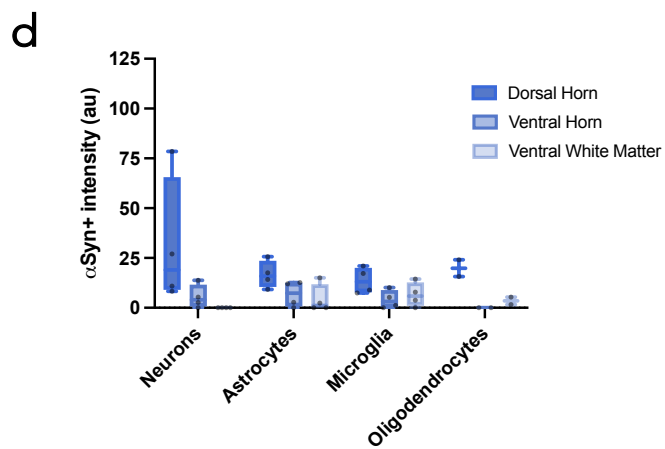
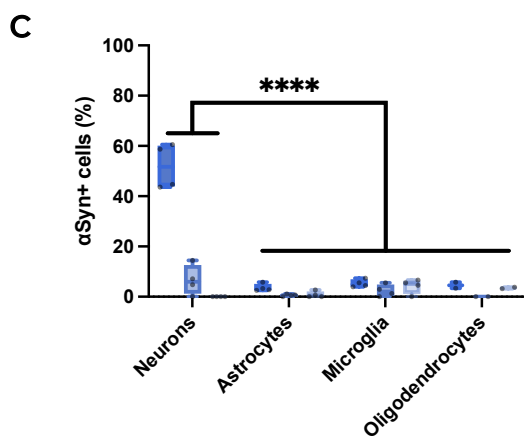
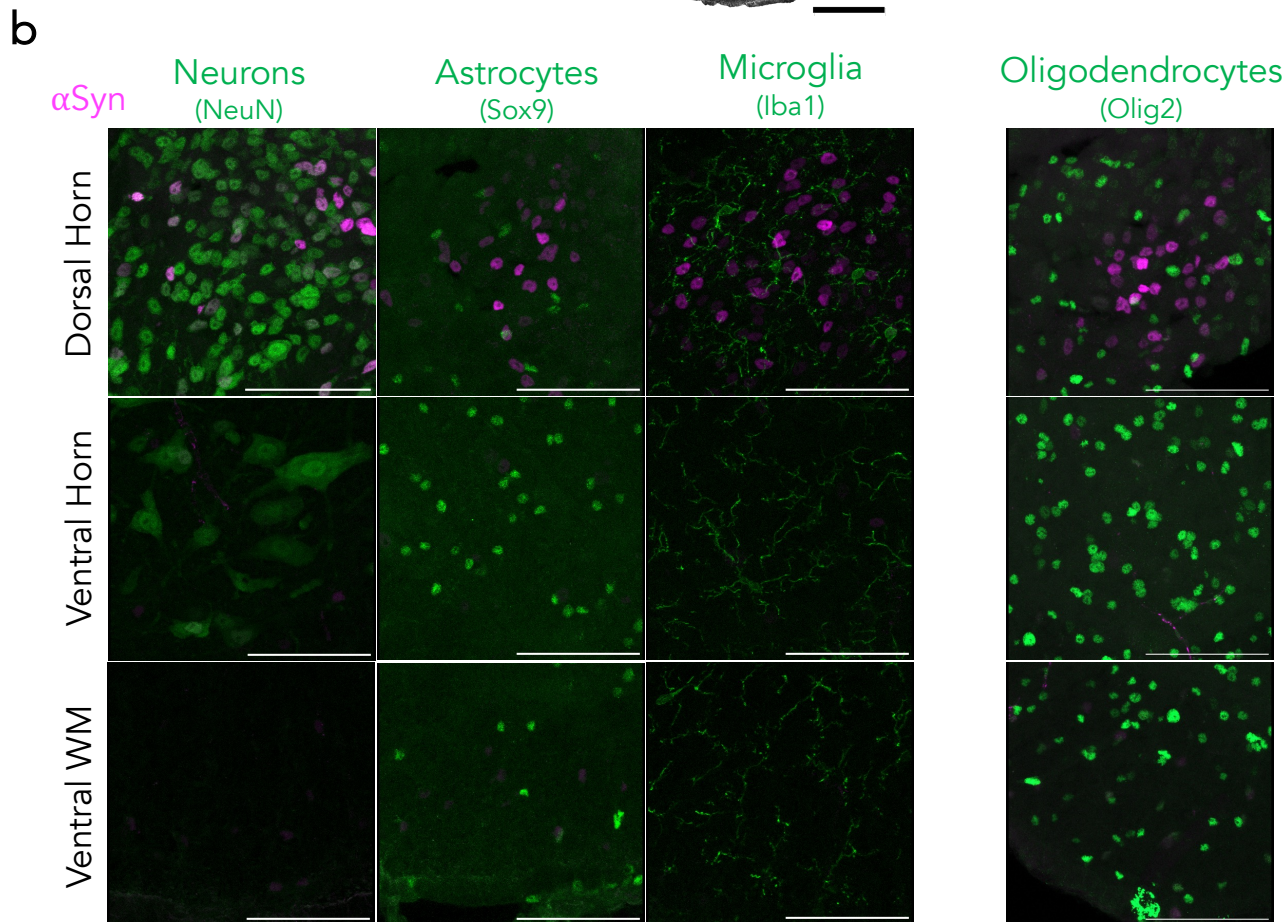
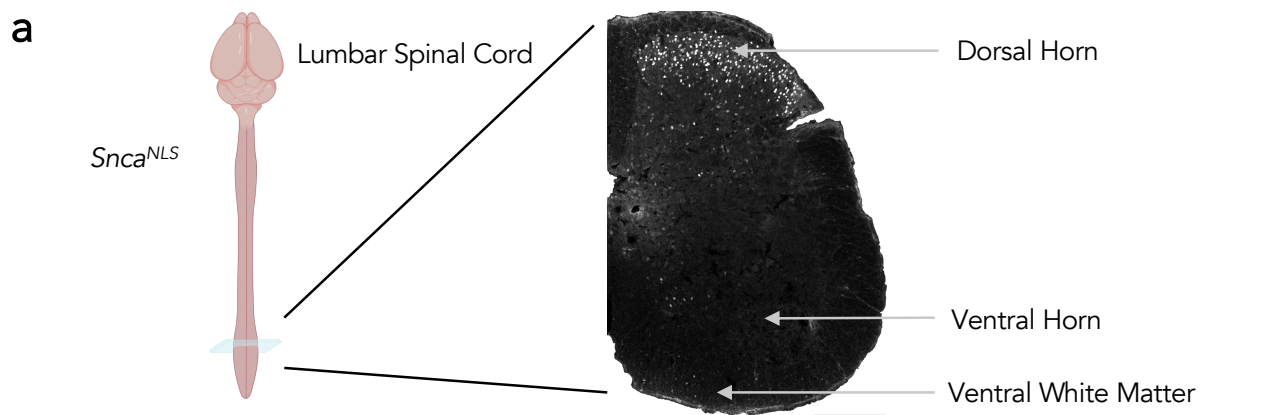
Supplementary Information



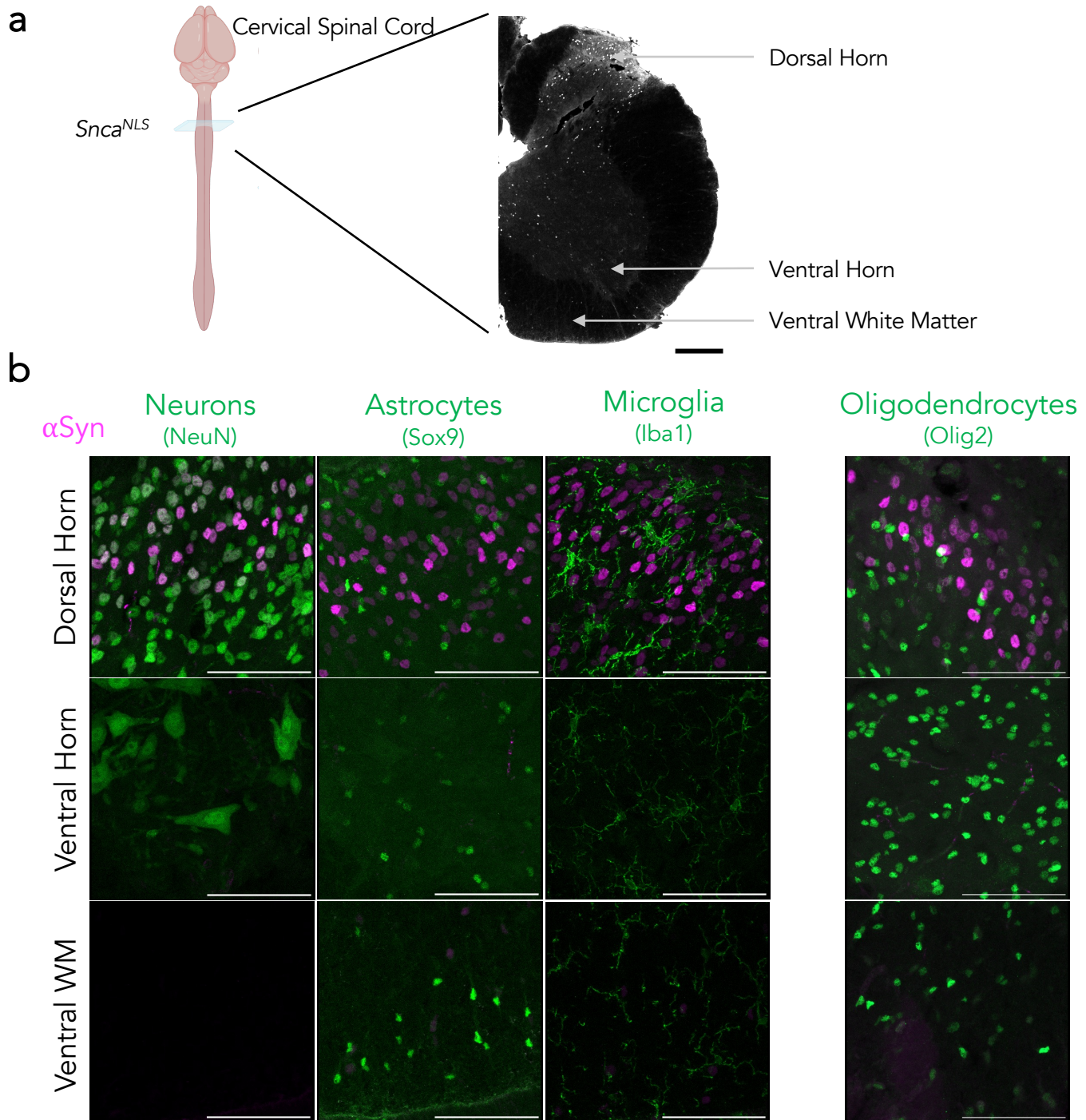
Supplementary Figure 1: Brain-wide heatmaps of Snca expression and α Syn protein density. **a)** Snca intensity from RNAScope of a wild-type mouse binned into low (upper panel), medium (middle panel), and high intensity (lower panel) prior to generating the heatmaps. **b)** α Syn staining intensity of a Snca^{NLS} mouse binned into low (upper panel), medium (middle panel), and high intensity (lower panel) prior to generating the heatmaps.



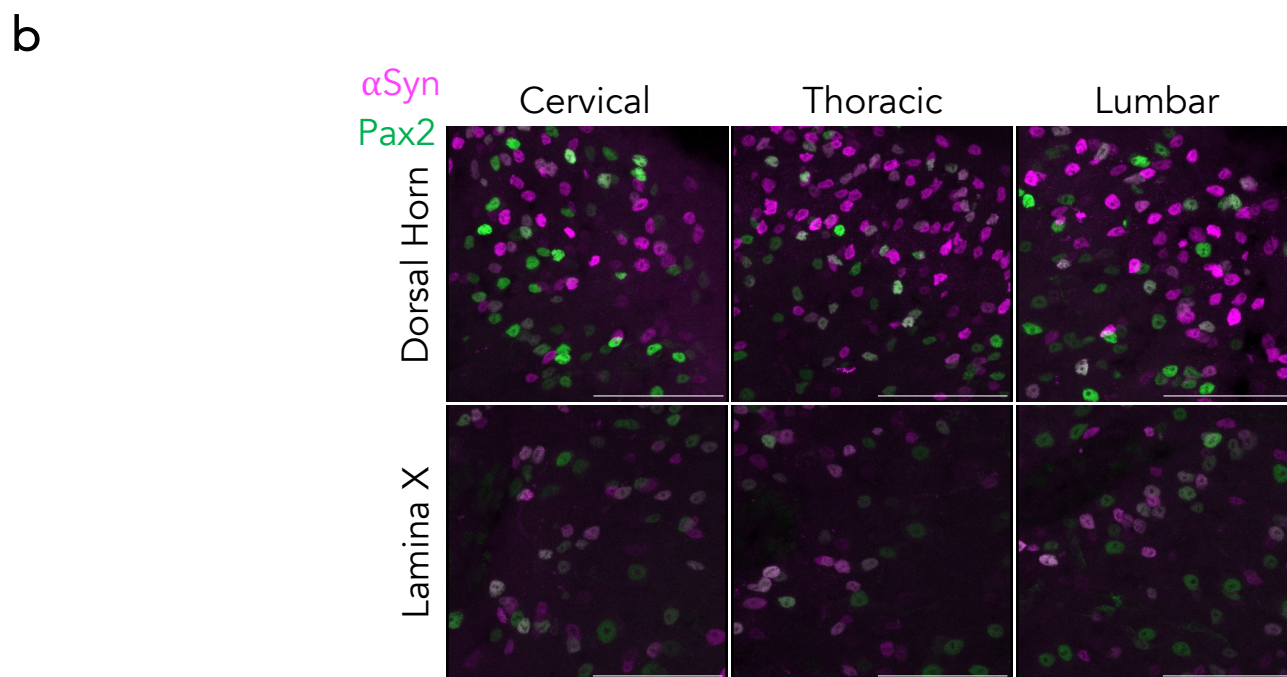
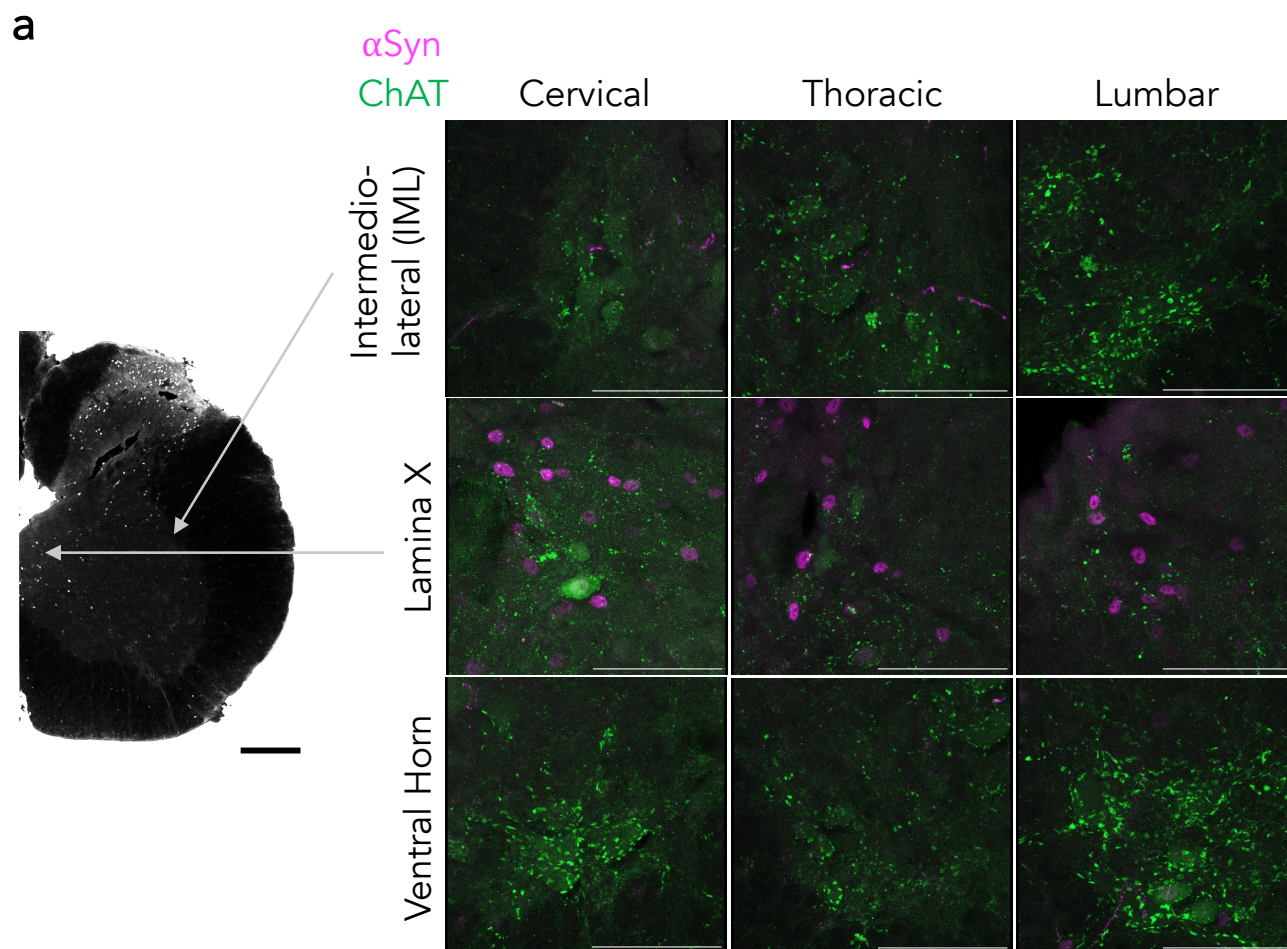
Supplementary Figure 2: Brain-wide atlas of α Syn protein density and intensity. **a)** Coronal atlas of α Syn DAB staining (left) and ilastik segmentation (right). **b)** Sagittal atlas of α Syn ilastik segmentation following DAB staining. **c)** Quantification of α Syn density in targeted brain regions. **d)** Quantification of α Syn⁺ density and intensity from whole brain staining and imaging. Plotted as box plots with min to max bars.



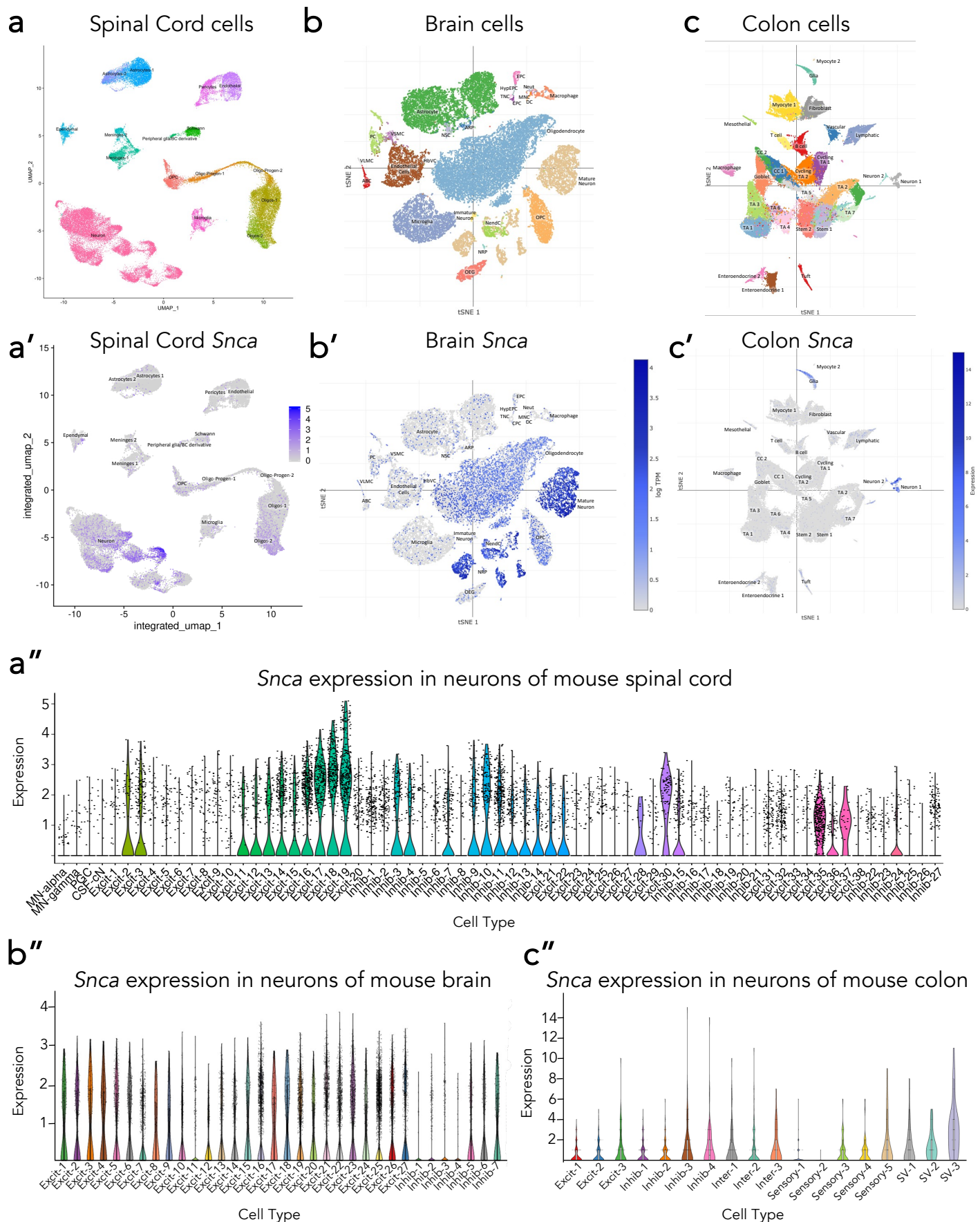
Supplementary Figure 3: α Syn density and intensity of the lumbar spinal cord. **a)** Coronal plane chosen to assess different regions of the lumbar spinal cord in $Snc\alpha^{NLS}$ mice (left) with annotations for the dorsal and ventral horns and ventral white matter (right) from a section stained for α Syn. Scale bar: 1,000 μ m. **b)** Merged micrographs from the dorsal horn (upper), ventral horn (middle), and ventral white matter (bottom) staining for neurons (left), astrocytes (middle left), microglia (middle right), and oligodendrocytes (right; different staining paradigm). From these, α Syn density (**c**) and intensity (**d**) were quantified. Scale bars: 75 μ m. Plotted as box plot with min to max bars. Two-way ANOVA with Tukey's post hoc, **** denotes $p < 0.0001$.



Supplementary Figure 4: α Syn staining of the cervical spinal cord. **a)** Coronal plane chosen to assess different regions of the lumbar spinal cord in *Snca^{NLS}* mice (left) with annotations for the dorsal and ventral horns and ventral white matter (right) from a section stained for α Syn. Scale bar: 1,000 μ m. **b)** Merged micrographs from the dorsal horn (upper), ventral horn (middle), and ventral white matter (bottom) staining for neurons (left), astrocytes (middle left), microglia (middle right), and oligodendrocytes (right; different staining paradigm). Scale bars: 75 μ m.



Supplementary Figure 5: α Syn staining patterns throughout the spinal cord with neuron-specific markers for ChAT and Pax2. **a)** Coronal plane chosen to assess cervical (left) thoracic (middle) or lumbar (right) Snca^{NLS} spinal cord in the intermediolateral (upper), lamina X (middle), and ventral horn (lower) co-stained for α Syn and ChAT. Scale bar or coronal plane: 1000 μ m. Scale bars for immunofluorescent images: 75 μ m. **b)** Coronal plane chosen to assess cervical (left) thoracic (middle) or lumbar (right) Snca^{NLS} spinal cord in the dorsal horn (upper) and ventral horn (lower) co-stained for α Syn and Pax2. Scale bars: 75 μ m.



Supplementary Figure 6: Mining scRNA-Seq datasets to explore α Syn expression patterns in the mouse spinal cord, brain, and colon. **a)** UMAP plot from the Harmonized Atlas of Mouse Spinal Cord with **a')** Snca expression pattern and **a'')** neuron-specific sub-clustering. **b)** tSNE plot from the Aging Mouse Brain dataset from the Broad Institute's Single Cell Portal with **b')** Snca expression pattern and **b'')** neuron-specific sub-clustering from the study Dissecting Cell-Type Composition and Activity-Dependent Transcriptional State in Mammalian Brains by Massively Parallel Single-Nucleus RNA-Seq. **c)** tSNE plot from the Human and Mouse Enteric Nervous System at Single Cell Resolution from the Broad Institute's Single Cell Portal with **c')** Snca expression pattern and **c'')** neuron-specific sub-clustering. ABC (arachnoid barrier cells), ARP (astrocyte-restricted precursors), CC (colonocyte), CPC (choroid plexus epithelial cells), CSF-cN (cerebrospinal fluid contacting neurons), DC (dendritic cells), EPC (ependymocytes), Excit- (excitatory neurons), HbVC (hemoglobin-expressing vascular cells), HypEPC (hypendymal cells), Inhib- (inhibitory neurons), Inter- (interneurons), MN (motor neurons), MNC (monocytes), NendC (neuroendocrine cells), Neut (neutrophils), NRP (neuronal-restricted precursors), NSC (neural stem cells), OEG (olfactory ensheathing glia), OPC (oligodendrocyte precursor cells), PC (pericytes), PGC (preganglionic cells), Sensory (sensory neurons), Stem (stem cells), SV (secretomotor/vasodilator neurons), TA (transit-amplifying cells), TNC (tanycytes), VLMC (vascular and leptomeningeal cells).

Supplementary Table 1: Antibodies used in this study with their application

	Antibodies (alphabetical)	Provider, Catalog #, RRID	Species	Application	Concentration
Primary	α Synuclein	BD Biosciences, 610787, RRID:AB_398108	Mouse	Immunofluorescence, α Syn ^{NLS} Atlas	1:2000
				Immunofluorescence, cell-specific staining & Immunohistochemistry, α Syn DAB Atlas	1:1000
		Cell Signaling Technology, 4179, RRID:AB_1904156	Rabbit	ENS immunofluorescence	1:250
	Calbindin D28K	Invitrogen, PA5-85669, RRID:AB_2792808	Rabbit	Immunofluorescence, cell-specific staining	1:1000
	Calretinin	Invitrogen, PA5-143562, RRID:AB_2942791	Chicken	Immunofluorescence, cell-specific staining	1:1000
	Choline acetyltransferase (ChAT)	EMD Millipore, AB144P, RRID:AB_2079751	Goat	Immunofluorescence, cell-specific staining	1:200
	DAPI	Millipore Sigma, D9542	-	Immunofluorescence, cell-specific staining	1:1000
	Flag (DDDDK)*	Abcam, ab1257, RRID:AB_299216	Goat	Immunofluorescence, α Syn 3D Atlas	4 μ g
	HuC/HuD	ThermoFisher Scientific, A-21271, RRID:AB_221448	Mouse	ENS immunofluorescence	1:100
	Iba1	Fujifilm Wako, 019- 19741, RRID:AB_839504	Rabbit	Immunofluorescence, cell-specific staining	1:1000
	NeuN	Millipore Sigma, 3881957, RRID:AB_10807954	Rabbit	Immunofluorescence, α Syn ^{NLS} Atlas	1:500
		Cell Signaling Technologies, 12943S, RRID:AB_2630395	Rabbit	Immunofluorescence, cell-specific staining	1:1000
		EnCor Biotechnology, MCA-1B7, RRID:AB_2572267	Mouse	Immunofluorescence, α Syn 3D Atlas	5 μ g
	Olig2	R&D Systems, AF2418, RRID:AB_2157554	Goat	Immunofluorescence, cell-specific staining	1:200
	Pax2	R&D Systems, AF3364- SP, RRID:AB_10889828	Goat	Immunofluorescence, cell-specific staining	1:200
	Sox9	Abcam, ab185966, RRID:AB_2728660	Rabbit	Immunofluorescence, cell-specific staining	1:1000
	Tuj1 (β III-tubulin)	Sigma-Aldrich, T8660, RRID:AB_477590	Mouse	ENS immunofluorescence	1:500
	Tyrosine hydroxylase	Millipore, AB152, RRID:AB_390204	Rabbit	Immunofluorescence, cell-specific staining	1:1000
Secondary	Donkey-anti-Goat Alexa Fluor 488	ThermoFisher Scientific, A32814, RRID:AB_2762838	Goat	Immunofluorescence	1:500
	Goat-anti-Mouse IgG (H+L) Alexa Fluor 488	Thermo Fisher Scientific, A-11001, RRID:AB_2534069			
	Goat-anti-Mouse IgG2b Alexa Fluor 568	Thermo Fisher Scientific, A-21144, RRID:AB_2535780	Mouse		
	Donkey-anti-Mouse Alexa Fluor 594	ThermoFisher Scientific, R37115, RRID:AB_2556543			
	Goat-anti-Rabbit IgG (H+L) Alexa Fluor 647	ThermoFisher Scientific, A-21244, RRID:AB_2535812	Rabbit		
	Goat-anti-Chicken IgY (H+L) Alexa Fluor 647	Thermo Fisher Scientific, A-21449, RRID:AB_2535866	Chicken		
	Goat-anti-Mouse IgG1 Alexa Fluor 488	Invitrogen, A21121, RRID:AB_2535764	Mouse	Immunofluorescence	1:1000
	Goat-anti-Rabbit Alexa Fluor 546	Invitrogen, A11010, RRID:AB_2534077	Rabbit		
Staining Kits	VectaStain Elite ABC Peroxidase Kit	Vector Laboratories, PK- 6102, RRID:AB_2336821	Mouse	Immunohistochemistry, α Syn DAB Atlas	-
	DAB Substrate Kit, Peroxidase HRP, with Nickel (3,3'-diaminobenzidine)	Vector Laboratories, SK- 4100, RRID:AB_2336382	-	Immunohistochemistry, α Syn DAB Atlas	-

*this antibody has since been discontinued

Chapter 5: General discussion and conclusion

5.1 Summary

PD is a devastating, multifaceted disease that affects millions of people worldwide. Uncovering the mechanisms of disease holds the potential to catalyze advancements in therapeutic development or the discovery of disease-modifying treatments, addressing the current deficit in such interventions.

Given the complexity of PD, we set out to initially characterize an understudied, yet important facet of disease: nuclear α Syn accumulation. Previous work identified α Syn as a central feature of PD, both through mutations and multiplications of the *SNCA* gene and cytoplasmic α Syn protein aggregation (Polymeropoulos et al., 1997; Spillantini et al., 1997; Singleton et al., 2003). However, recent work noted an accumulation of nuclear α Syn in disease, and that this nuclear accumulation could contribute to disease pathogenesis (Siddiqui et al., 2012; Rousseaux et al., 2016; Chen et al., 2020; Koss et al., 2022).

Understanding the consequences of α Syn nuclear localization is a central theme in this thesis. However, parsing out the underlying mechanism behind nuclear α Syn toxicity has proven difficult since the models have additional disease-associated factors, including α Syn mutations, PTMs, or aggregation. Previously, groups targeting the nuclear localization of α Syn relied on overexpressing α Syn with a NLS tag, or mutating α Syn, both of which (overexpression and mutation) foster a more aggregate-prone α Syn (Masliah et al., 2000; Specht et al., 2005; Kontopoulos et al., 2006; Fares et al., 2014). These confounding factors led us to generate the

Sncα^{NLS} mouse to isolate the contribution of accumulating nuclear-localized αSyn in disease. Therefore, using the *Sncα^{NLS}* model proved useful for sequestering nuclear-specific mechanisms of disease.

In addition to understanding the nuclear accumulation of αSyn, another PD research focus has been the propagation of αSyn pathology from cell-to-cell. Initial studies found αSyn aggregates, LBs and LNs, to spread throughout PD brains in a prion-like manner and their pattern correlated to disease severity (Braak et al., 2003). Further understanding of this spreading revealed that aggregate-prone αSyn can “seed” endogenous αSyn to take on an aggregate-prone conformation, thereby propagating pathological protein (Desplats et al., 2009; Luk et al., 2009; Volpicelli-Daley et al., 2011; Dadgar-Kiani et al., 2022). Again, the *Sncα^{NLS}* model provided an alternate tool to study the cell-specific topography of αSyn protein due to the clear nuclear localization of αSyn making it easier to identify cells-of-origin. Understanding the cells which harbor different concentrations of αSyn may enlighten the neural circuits underlying αSyn spreading, giving further insight into cellular vulnerability in PD.

This thesis aims to gain a better understanding of the consequences of nuclear αSyn accumulation, the protein-protein interactions behind αSyn nuclear-specific neurotoxicity, and to catalog the topography of endogenous αSyn throughout the central and peripheral nervous systems. Uncovering these details of αSyn dysfunction and localization will enable a more encompassing view of PD, provide a better pre-clinical model for nuclear αSyn, and reveal new avenues for disease-modifying therapeutics.

5.2 Principal Findings

In this dissertation, we begin to parse the mechanisms for nuclear α Syn toxicity and provide a framework for studying endogenous α Syn cell-specific localization. In chapter 2, we began by developing a new model to study nuclear α Syn accumulation by knocking in an NLS-Flag tag onto the C-terminus of endogenous α Syn in mice. Upon aging, we noted a significant age-dependent decline in motor function, paired with reduced fecal output and early lethality. The motor dysfunction was most prominently observed during the rotarod test and suggests the mice may have difficulty with gait and balance. While this is largely thought to be due to the dopaminergic neurodegeneration in PD, this finding suggests that altered α Syn dynamics, specifically within the nucleus, could be contributing to these symptoms. Aged mice also exhibited significant cortical thinning specific to layer V/VI of the motor cortex, and increased pyknotic cells, suggesting a possible cause for the motor dysfunction. Additionally, we performed mass spectrometry on the mid-aged mouse cortex and found a decrease in proteins involved in dopamine signalling, which could also be contributing to the motor deficits. We also noted constipation phenotypes in the mice at later age timepoints, although constipation is viewed as a prodromal symptom of PD. However, nuclear α Syn accumulation may act synergistically with other factors of disease, like α Syn aggregation, to induce earlier constipation symptoms in people with PD. Interestingly, none of these phenotypes were present in *Snc α ^{KO}* mice, supporting a toxic gain-of-function of nuclear α Syn.

In chapter 3, we sought to identify novel α Syn nuclear interacting proteins to decipher the mechanism behind nuclear α Syn toxicity. We used the proximity labelling assay, TurboID, to identify proteins within a ~40 nm radius of mislocalized α Syn. Here, we found significant

enrichment for RNA binding proteins specific to the $\alpha\text{Syn}^{\text{NLS}}$ condition, compared with αSyn and $\alpha\text{Syn}^{\text{NES}}$. One of the top hits, Luc7l, was validated to interact with nuclear-specific αSyn via bimolecular fluorescence complementation and co-immunoprecipitation assays. Further analysis of αSyn -Luc7l dynamics uncovered a potential stabilization of αSyn protein upon Luc7l overexpression. Interestingly, LUC7L protein levels are elevated in PD, suggesting a possible mechanism contributing to αSyn overexpression then aggregation via LUC7L interaction. However, this interaction warrants further investigation to determine if LUC7L-mediated αSyn overabundance is due to protein stabilization or *SNCA* overexpression, and if this contributes to αSyn aggregation in disease.

In chapter 4, we addressed the cell type-specific expression of αSyn to gain insight into the pattern of prion-like αSyn spreading. Repurposing young adult *Snca*^{NLS} mice, we harnessed nuclear αSyn localization to delineate cells which harbour different αSyn protein dosages. Unsurprisingly, we noted most neurons throughout the brain contain αSyn , with a significantly smaller pool of astrocytes, microglia, and oligodendrocytes that had detectable levels of αSyn . For the most part, brain regions with a high αSyn^+ density correlated to brain regions that have a large burden of LBs in PD post-mortem brains. However, within some neuronal populations, we noted some discrepancies both in regions with a high density of αSyn^+ but no documented LB pathology, and no/low αSyn^+ density but prominent LB pathology. This suggests that while endogenous αSyn is important for propagating αSyn pathology, it is not the only factor in cell-specific vulnerability. Alternatively, it could be that in disease, *SNCA* is expressed in cells that do not normally express *SNCA*, thereby creating an environment that has the capacity to propagate αSyn pathology. The primarily neuron-specific localization of αSyn extended to the

spinal cord, in the dorsal horn and lamina X, and the retina, in the ganglionic cell and inner plexiform layers, harboured the most α Syn⁺ cells. Expanding from the central nervous system to the gut, we noted all myenteric neurons in the stomach and duodenum had detectable α Syn protein, but there was a high heterogeneity in signal intensity, suggesting potential neuron sub-type-specific expression. Further investigation into the pattern of high versus low α Syn⁺ cells in the gut may provide clarity on the α Syn spreading hypothesis from the gut to the brain and could enable a more mechanistic understanding of the gastrointestinal symptoms associated with PD. Altogether, this atlas of α Syn topography provides a base for future comparison models examining α Syn expression or localization changes to various disease-relevant insults (e.g. genetic or viral perturbations). Moreover, it can be used to explore the multiple hit hypothesis in identifying factors that contribute to α Syn spreading and cell-specific vulnerability.

5.3 Future Directions

The results presented in this dissertation provide new insight into the possible mechanism(s) for nuclear α Syn neurotoxicity. Firstly, the *Snca*^{NLS} mouse highlights the consequences of chronic nuclear accumulation of α Syn. However, we have only begun to understand the mechanisms behind this neurotoxicity. Previous reports have shown the binding capacity of α Syn to DNA, however we did not note significant changes to the transcriptome in these mice (not shown). Moreover, we initially aimed to investigate the DNA binding capacity of α Syn in the *Snca*^{NLS} mice using the Cleavage Under Target and Release Under Nuclease (CUT&RUN) protocol, however we did not detect significant enrichment of specific α Syn-DNA binding (data not shown). Although this α Syn-DNA binding may be important developmentally and in cycling

cells, further research is needed to determine if and how α Syn is binding to DNA with age, especially in this model where α Syn does not have a propensity for aggregation nor disease-associated PTMs.

Alternatively, when α Syn accumulates in the nucleus, it could be gaining and/or preventing nuclear-specific protein interactions to contribute to disease. We have postulated in chapter 3 that α Syn may be disrupting the spliceosome through its interaction with many spliceosome/RNA binding proteins, including Luc7l. Although the characterization of the α Syn-Luc7l interaction is still in its infancy, the multiple orthogonal validation methods suggest that the interaction is indeed real. This, coupled to the positive effect of Luc7l overexpression on α Syn paints a tantalizing picture of a new level of nuclear α Syn regulation. Further exploring how nuclear α Syn fits within this context will be imperative in understanding the toxic role it plays in disease. Additional investigation is also needed to determine if the Luc7l-mediated α Syn stability is existing on the protein level, or if Luc7l overexpression is leading to a higher rate of *SNCA* expression. Moreover, identifying the binding regions for both proteins will help elucidate the functional consequences of this interaction – i.e. determine if Luc7l is binding the N-, NAC, or C-terminus of α Syn. Validation of additional spliceosome/RNA binding proteins will be imperative in understanding the relationship between α Syn and this protein group, with the goal of generating novel therapeutic targets for PD. For example, if nuclear α Syn toxicity is driven by its interaction with these proteins, either alone or in a larger complex, then disrupting this interaction with a peptide could be an attractive therapy.

We have also presented the framework for others to build upon the understanding of α Syn cell-to-cell spreading through the *Snca*^{NLS} atlas. This atlas highlights the cell-specific intensity and density of α Syn⁺ cells throughout the central and peripheral nervous system. Importantly,

α Syn localization was generally expected based on RNA studies and post-mortem analysis of PD tissue however there was imperfect overlap with *Snc*a RNA, α Syn in the *Snc*a^{NLS} mice, and in α Syn pathology from post-mortem brains. Future work can explore why these differences exist and the importance of these discrepancies in disease – specifically with regards to cell-specific vulnerability or *why* α Syn pathologically spreads from cell-to-cell. Follow-up studies can also add various disease-relevant insults to the *Snc*a^{NLS} mice and compare α Syn topography against this original atlas. Some of these insults can include traditional, well-known PD insults such as PFFs, environmental toxins (e.g. rotenone, paraquat and permethrin), and aging. This will provide insight into how α Syn topography may be changing with disease and may better recapitulate where LBs are found throughout the PD brain. Alternatively, this atlas can be used to design studies targeting α Syn-rich or α Syn-poor circuits, especially when investigating cell-specific vulnerability.

In conclusion, the results presented in this thesis offer insights into the effects of chronic nuclear α Syn accumulation, initiate the exploration into the underlying mechanisms behind this neurotoxicity, and unveil the endogenous cell-specific protein localization of α Syn. Collectively, these findings contribute to enriching our existing understanding of α Syn in both healthy and disease contexts, while also introducing a novel mouse model for targeting investigations into the nuclear accumulation of α Syn. By targeting these crucial aspects of PD, we uncover new avenues for understanding the intricacies of α Syn protein dynamics. Expanding upon our grasp of cell-specific vulnerability and the role of nuclear α Syn, we envision the possibility of identifying novel therapeutic targets to mitigate the impacts of disease. As we continue to expand our knowledge, leveraging these discoveries may lead to transformative breakthroughs in the

field, offering hope for improved interventions and ultimately, better outcomes for individuals affected by α -synucleinopathies.

Appendix I

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Appendix II

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Constitutive nuclear accumulation of endogenous alpha-synuclein in mice causes motor impairment and cortical dysfunction, independent of protein aggregation

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Publication: Human Molecular Genetics

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A topographical atlas of α -synuclein dosage and cell type-specific expression in adult mouse brain and peripheral organs

Author: Haley M. Geertsma Benjamin R. Arenkiel et al

Publication: npj Parkinson's Disease

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Convergent systems-based approaches identify a role for OCIAD1 in Alzheimer's disease

[Haley M. Geertsma](#)^{a,b} and [Maxime W.C. Rousseaux](#)^{b,c,d,*}

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SPRINGER NATURE

Assessment of Dopaminergic Neurodegeneration in Mice

Author: Haley M. Geertsma, Konrad M. Ricke, Maxime W. C. Rousseaux

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Additional Data



Characterizing the differential distribution and targets of Sumo1 and Sumo2 in the mouse brain

Author:

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Appendix III

Appended articles

Geertsma HM, Rousseaux MWC. Convergent systems-based approaches identify a role for OCIAD1 in Alzheimer's disease. **EBioMed**, Volume 52, 102627, February 2020, <https://doi.org/10.1016/j.ebiom.2020.102627>

Contribution: I helped to write and edit this commentary. My overall contribution to this paper was about 70%.



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Commentary

Convergent systems-based approaches identify a role for OCIAD1 in Alzheimer's disease

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Alzheimer's disease (AD) affects approximately 30–35 million individuals worldwide, posing a tremendous medical and fiscal burden on society. With life expectancy on the rise, the World Health Organization estimates that the number of people living with AD is predicted to triple by 2050. While its pathogenesis remains elusive, targeting Amyloid β ($A\beta$) pathology – a hallmark of the disease – in individuals with AD has been a primary focus for industry [1]. Recent failures of clinical trials targeting $A\beta$ have increased the need for a better understanding of AD pathogenesis, particularly its earliest events. Though it is well appreciated that $A\beta$ accumulates in the brains of individuals with AD, the initial consequences of hyperamyloidosis, one of the earliest pathological changes of this disease, remains poorly understood [2]. It is plausible that a better understanding of the earliest phenomena downstream of hyperamyloidosis will help elucidate disease pathogenesis and give rise to novel therapeutic avenues.

There are increasing numbers of transcriptomic and proteomic datasets from both human AD cases and mouse models that can be mined, through bioinformatics followed by functional validation in orthogonal cellular platforms, to yield novel insights into the disease pathogenesis. In this issue of *EBioMedicine*, Li and colleagues contribute to these datasets, devise a pipeline for the identification of novel AD-centric pathways through the integration of multiple “omics” datasets, and elucidate a novel role for OCIAD1 (Ovarian Cancer Immunoreactive Antigen Domain Containing 1) in AD [3]. To begin, they identify key regulatory mechanisms downstream of amyloidosis by performing proteomic assays on two established mouse models of AD, while concurrently comparing gene expression profiles of

vulnerable (entorhinal cortex and hippocampus) to less vulnerable (visual cortex) brain regions of sporadic AD patients. The convergence of these datasets yields three factors associated with disease development, one of which, OCIAD1, is upregulated in disease states. In exploring the relationship between OCIAD1 and disease development, Li et al. find that decreasing GSK3 β , a key kinase in AD [4], also decreases OCIAD1 levels in cellular models and that, conversely, elevated OCIAD1 levels exacerbate multiple cellular stress responses. Additionally, by examining the protein-protein interaction networks of OCIAD1, they delineate a relationship between BCL-2, OCIAD1, and BAX in mitochondrial-associated neurodegeneration. Together, the authors conclude that OCIAD1 is a novel neurodegeneration-associated factor in the early stages of AD.

Several steps remain before moving this target forward for pre-clinical trials. First, given that OCIAD1 is ubiquitously expressed throughout the body [5], a careful look at the consequences of its loss in model organisms may shed important light on its native function. Earlier this year, a report suggested that mice lacking *Asrij* (the mouse *OCIAD1* ortholog), are viable and fertile, though progressively accumulate hematological deficits over time [6]. Thus, inhibition of OCIAD1 in humans may require careful regulation in the context of its tissue locale, particularly in the context of an aging population. To wit, a first step forward will be to test whether decreasing *Asrij* is sufficient to mitigate neurodegenerative phenotypes in mouse models of AD. Second, given that a treatment targeting OCIAD1 would be chronic in nature, traditional pharmacology (as opposed to antibody- or antisense oligonucleotide-based approaches) would be a promising approach. As such, it will be critical to identify the specific pathological mechanism of OCIAD1 in order to inhibit its toxicity in AD. Given its potential role in scaffolding proteins as well as regulated mitochondrial metabolism, this may prove to be difficult [7–9]. Alternatively, targeting the regulators of OCIAD1 or its downstream effectors may be additional routes for intervention; though the efficacy of inhibiting targets such as GSK3 β while maintaining specificity remains a challenge.

An additional avenue of future investigation will be the use of OCIAD1 as a potential biomarker for AD. Li and colleagues found that OCIAD1 protein levels in the hippocampus correlate with increased pathological staging. To expand on this finding, it will be important to mine the growing body of AD sample/tissue

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E-mail address: max.rousseau@uottawa.ca (M.W.C. Rousseaux).<https://doi.org/10.1016/j.ebiom.2020.102627>2352-3964/© 2020 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license. (<http://creativecommons.org/licenses/by-nc-nd/4.0/>)

repositories (e.g. ADNI, the Alzheimer's Disease Neuroimaging Initiative [10]) to determine if OC1AD1 is found in blood or cerebral spinal fluid and whether changes in its levels correlate with disease status. Using OC1AD1 as an early disease biomarker could help track disease progression and lead to an earlier marker of disease onset to aid in symptom management and future disease-modifying treatment for individuals living with AD.

Declaration of Competing Interest

None declared.

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Contribution: I helped with writing and editing the protocol and provided images for the figure.

My overall contribution to this paper was about 50%.



Chapter 10

Assessment of Dopaminergic Neurodegeneration in Mice

Haley M. Geertsma, Konrad M. Ricke, and Maxime W. C. Rousseaux

Abstract

Neuron death is a key feature of neurological disorders like Alzheimer's or Parkinson's disease (PD). As a result, analysis of neurodegeneration is often considered a central experiment in the postmortem characterization of preclinical PD animal models. Stereology provides a precise estimate of particles, like neurons, in three-dimensional objects, like the brain, and is the gold standard quantification approach for the assessment of neuron survival in neurodegenerative disease research. Here, we provide a detailed step-by-step guide for the quantification of dopaminergic neurons in the substantia nigra pars compacta, a brain area prone to neuron loss in PD. In addition, we outline the protocol for the analysis of the dopaminergic terminals in the striatum, the projection area of midbrain dopaminergic neurons, as a readout for the integrity of the nigrostriatal projections.

Key words Neurodegeneration, Parkinson's disease, Mouse models, Substantia nigra pars compacta, Striatum, Immunohistochemistry, Stereology, Optical fractionator, Physical fractionator, Optical densitometry

1 Introduction

Loss of dopaminergic neurons (DaNs) in the substantia nigra pars compacta (SNc) is a neuropathological hallmark of Parkinson's disease (PD). Death of SNc DaNs is accompanied by the disruption of the nigrostriatal pathway and the consecutive imbalance of the basal ganglia circuitry [1–3]. These cerebral alterations result in the typical motor symptoms of patients, rendering this neuron population a frequent target for analysis in PD research [4]. Loss of DaNs is also observed in a midbrain region neighboring the SNc, the ventral tegmental area (VTA). However, cell death is more pronounced in the SNc compared to the VTA which is reflected by the differential loss of dopaminergic striatal projections, originating from these two neuron populations [2–9]. Degeneration of the nigrostriatal system is frequently modeled in PD animal models [10] using chemical insults (e.g., by the injection of

6-hydroxydopamine [11], 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [12] or rotenone [13]) or by genetic manipulation [14–17], typically followed by the quantification of surviving SNc DaNs in the dissected brain by histological procedures.

“Semiquantitative” histological approaches for the estimation of neuron numbers using representative brain sections can be imprecise and may result in the misinterpretation of results, as the selection of sections may be performed in a nonrandom fashion, resulting in sampling bias [18]. Furthermore, substantial parts of the analyzed brain area may be neglected, falsifying the outcome of the estimation. Stereological quantification strategies overcome these pitfalls by providing an unbiased, systematic cell number assessment, encompassing the entire region of interest in the brain [18, 19].

We present a detailed guide for the quantification of neuron numbers and their process density, which we have applied to count SNc DaNs (and their respective striatum projections) in multiple PD mouse models [14, 20–22]. This protocol includes the collection and processing of the brain tissue, the immunohistological (IHC) procedures and the stereological quantification of neurons in the SNc using the optical fractionator approach. We also describe an alternative to the optical fractionator, the physical fractionator, which can be performed with open-source software and outline the quantification of striatal projections of SNc DaNs as a fast and complimentary readout for the deterioration of the nigrostriatal system in PD mouse models.

2 Materials

Prepare all solutions using purified deionized water and analytical grade reagents. Prepare and store reagents at room temperature (unless stated otherwise).

2.1 Reagents Required (in Order of Appearance)

1. 65 mg/mL Euthanyl (pentobarbital sodium).
2. 10× PBS: 25.6 g sodium dibasic, 80 g sodium chloride, 2 g potassium chloride, 2 g potassium monobasic in water with a total volume of 1 L.
3. 4% Paraformaldehyde (PFA): 40 g PFA in 1 L water (Heat the water to 60 °C and slowly raise the pH until the PFA is dissolved. Then adjust the pH value to 6.9 and filter the solution through Whatman™ filter paper (150 mm diameter) using gravitational force (*see Note 1*)).
4. 10% sucrose + 0.02% NaN₃: 100 g sucrose, 2 mL 10% NaN₃ in water with a total volume of 1 L.
5. 20% sucrose + 0.02% NaN₃: 200 g sucrose, 2 mL 10% NaN₃ in water with a total volume of 1 L.

6. **30% sucrose + 0.02% NaN₃**: 300 g sucrose, 2 mL 10% NaN₃ in water with a total volume of 1 L.
7. Isopentane (also known as D-methylbutane).
8. 1× PBS + 0.02% NaN₃: 2 mL 10% NaN₃ in 100 mL 1× PBS.
9. 0.9% H₂O₂: 30 µL 30% H₂O₂ in 1 mL 1× PBS.
10. VECTASTAIN[®] Elite ABC Kit Peroxidase (HRP), PK-6101, Vector Laboratories.
11. DAB Substrate Kit, Peroxidase (HRP), with Nickel, (3,3'-diaminobenzidine), SK-4100, Vector Laboratories.
12. 50% ethanol: 500 mL 100% ethanol in water with a total volume of 1 L.
13. 70% ethanol: 700 mL 100% ethanol in water with a total volume of 1 L.
14. 90% ethanol: 900 mL 100% ethanol in water with a total volume of 1 L.
15. 95% ethanol: 950 mL 100% ethanol in water with a total volume of 1 L.
16. (100%) ethanol.
17. Xylenes.
18. Permount[™] (or other nonaqueous mounting medium).
19. Sodium citrate buffer: 2.94 g sodium citrate, 0.5 mL Tween 20 in 1× PBS in a total volume of 1 L. Adjust the pH value to 6.

2.2 Equipment and Consumables (in Order of Appearance)

1. Syringes (1 mL, 10 mL).
2. Needles (27 G × 1/2").
3. Styrofoam block.
4. Forceps (curved forceps for brain dissection (e.g., FST[™] 11009-13), straight forceps (e.g., FST[™] 11002-25) or a perforated surgical spoon for brain freezing).
5. Scissors (different sizes depending on personal preference).
6. Specimen vial (or 15 mL conical tube).
7. Dry ice.
8. Thermometer.
9. Paper tissue (e.g., Kimwipes[™]).
10. Aluminum foil.
11. Cryostat (Leica MICROSYSTEMS CM1850).
12. O.C.T. (Optimal Cutting Temperature Compound).
13. Microtome blades.
14. Fine brush.

15. Well plate (24-well plate for the collection of sections, 12-well plate for the staining).
16. PARAFILM®.
17. Fisherbrand™ Superfrost™ Plus Slides.
18. Super PAP Pen.
19. Coverslips.
20. Brightfield microscope equipped with a motorized specimen stage for optical fractionator (Leica MICROSYSTEMS DMRXE microscope) and with a computer with Stereo Investigator® software (MBF Bioscience, version 11.06.2).
21. Spreadsheet and statistical analysis software (Microsoft Excel, GraphPad Prism).
22. Tissue processor (Leica MICROSYSTEMS ASP300).
23. Tissue embedding station (Leica MICROSYSTEMS EG1150H).
24. Microtome (Leica MICROSYSTEMS RM2125 RTS).
25. Water bath (Leica MICROSYSTEMS HI1210).
26. Automated microscopy system (Leica MICROSYSTEMS SCN400) and slide viewing software (Leica MICROSYSTEMS SlidePath Gateway).
27. Image stitching software (Microsoft Image Composite Editor).
28. Image editing software (Adobe Photoshop or GNU Image manipulation Program).
29. National Institutes of Health (NIH) Image J (1.52p).

3 Methods

Carry out all procedures at room temperature unless stated otherwise.

The following descriptions are primarily intended for the assessment of SNc DaNs numbers using the optical fractionator approach performed after immunohistochemical staining on free-floating coronal cryosections of the mouse brain.

3.1 Brain Dissection and Processing

1. Anesthetize the mouse by intraperitoneal injection of euthanyl: Injection of 10 mL/kg body weight of euthanyl solution at 65 mg/mL results in an anesthetized state with surgical tolerance (loss of pedal withdrawal reflex) minutes after injection while remaining cardiac function (as per uOttawa ACC-4 Good Practice Guide to the Administration of Substances). Restrain the mouse and inject the euthanyl with a needle into the lower abdomen (*see Note 2*). Place the mouse into its cage

until a state of deep anesthesia and surgical tolerance is reached (loss of righting, pedal withdrawal and lid reflexes). Alternative anesthetics can be used. Please, refer to your local animal health and ethics authorities, as well as veterinarians for guidelines to avoid pain and distress of animals.

2. After reaching surgical tolerance (*see* **Note 3**), lay the mouse on its back with its ventral side exposed on a block of Styrofoam and pin down the limbs using needles. Cut through the abdominal skin and the peritoneum with scissors from caudal to cranial without damaging the internal organs (the blunt end of dissection scissors should be inside the cavity and the sharp end at the exterior) and continue to cut through the rib cage in a “V” shape enough to create a flap that can be pinned back (*see* **Note 4**). Locate the heart and its left ventricle.
3. Insert the needle (27 G $\times$ 1/2”) of a syringe containing ~10 mL of 1 $\times$ PBS into the left ventricle. Do not pierce the interventricular septum. Make a small (~2 to 3 mm) incision in the right atrium of the heart and slowly inject the 1 $\times$ PBS into the left ventricle, ensuring to not introduce bubbles during the procedure. This process replaces the blood with 1 $\times$ PBS and should take roughly 1.5–2 min. Remove the syringe from the heart (*see* **Note 5**).
4. Insert the needle of a syringe with ~10 mL of 4% PFA (*see* **Note 6**) into the same puncture of left ventricle without piercing the interventricular septum and slowly inject the 4% PFA to fix the whole body.
5. Remove the needle tips that were pinning the limbs down and flip the mouse over to an upright position (exposing the dorsal side). If the mouse is rigid and the limbs do not sag, the body is completely fixed.
6. Decapitate the mouse just posterior to the ears (The remaining body can be used for further histological analysis). Using scissors, cut the skin on the top of the head from the incision site to the eyes and fold the skin flaps back to expose the skull. Make a small cut through the skull in between the eye sockets and carefully remove fat deposits from around the skull. Remove the foramen magnum by pulling it up and twisting it from side-to-side until it begins to lift and separate from the skull. Insert the scissors next to the spinal cord, cut laterally and peel back the two resulting flaps of the skull. Insert the scissors above the exposed cerebellum (*see* **Note 7**) and carefully cut anteriorly until the cut between the eye sockets is reached. Using forceps, fold back the skull, exposing the brain (do not to damage the brain). Spoon the brain out of the skull using forceps or other dull tools and drop the brain in a specimen vial with 4% PFA. Continue with the remaining mice. Store the brains at 4 °C for 24–48 h until further processing.

7. Cryoprotect the brains in 10% sucrose + 0.02% NaN₃ solution at 4 °C for 24–48 h, followed by 20% sucrose + 0.02% NaN₃ solution at 4 °C for 24–48 h and 30% sucrose + 0.02% NaN₃ solution for a minimum of 24 h.
8. Chill isopentane in a beaker on dry ice to –35 to –45 °C. Decant the 30% sucrose + 0.02% NaN₃ solution from the specimen vial and remove excess moisture from the brain using fine paper tissue. Gently lower brain in chilled isopentane with dull forceps and freeze brain for 60 s. Using prechilled (dry ice) forceps or a perforated surgical spoon, remove the frozen brain from isopentane and quickly wrap it in (prelabeled) aluminum foil. Store the brain on dry ice and continue with the remaining brains (*see Note 8*).
9. Store the brains at –80 °C. In our experience, brains for IHC can be stored at –80 °C for several years.
10. Set the cryostat chamber and the microtome to –20 °C. Once the temperature is reached, place the brains in the cryostat chamber for ~30 min to acclimatize the tissue to the temperature. In the cryostat chamber, mount the brain on the cryostat chuck using O.C.T. (Optimal Cutting Temperature Compound): Place the chuck in its holder in the chamber and apply little (2–3 mL) of O.C.T. (stored at RT) on it. Quickly mount the brain with its rostral end (cerebellum and medulla) in the O.C.T. in a fixed orientation (using chilled forceps) and completely engulf it with O.C.T. until the O.C.T. is hardened (the O.C.T. will turn white). Mount the chuck in the spindle of the microtome, the lateral side of the brain facing the knife and the dorsal side facing to the left, and section the brain at a thickness of 40 µm. In this orientation the brain will be cut in coronal sections from rostral to caudal. Try to avoid the loss of the brain sections as stereological quantification requires serial sections (*see Note 9*). Cryosectioning should be performed with a clean, dry, chilled (–20 °C), sharp blade and antiroll glass without notches. All cut material can be saved for histology; however, for the analysis of the nigrostriatal system, sections from the striatum (Bregma 1.70–0.62 mm) and the SNc (Bregma –2.54 to –3.88 mm [23]) have to be collected (*see Note 7* and Fig. 1). Striatal sections can be cut at a thickness of 20 µm for a higher resolution of striatal micrographs. Section thickness should be set back to 40 µm after having reached the caudal outline of the striatum, as this thickness is used for stereological quantification of midbrain DaNs using the optical fractionator (*see Note 10*). Using a fine brush, carefully transfer the section to a 24-well plate with 1× PBS + 0.02% NaN₃. Seal the plate with PARAFILM[®] for storage at 4 °C. Sections can be stored at for several months (*see Note 11*).

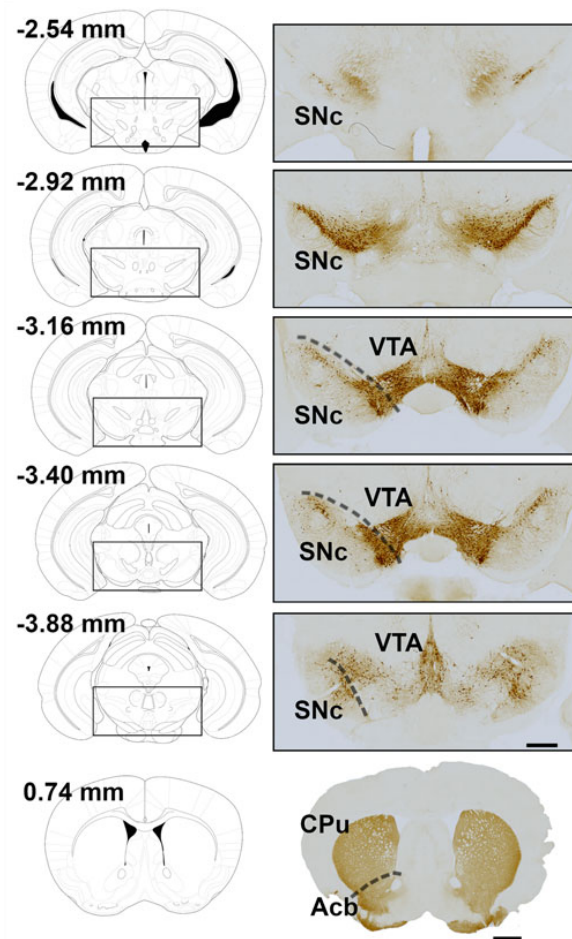


Fig. 1 The nigrostriatal system of the mouse brain. The substantia nigra pars compacta (SNc) is localized between Bregma -2.54 mm and -3.88 mm (left, schemes modified from [23]). SNc dopaminergic neurons (DaNs) can be visualized by tyrosine hydroxylase antibody staining (right, as details of the schemes to the left, $40\text{ }\mu\text{m}$ sections, $500\text{ }\mu\text{m}$ scale bar). In Parkinson's disease (PD) patients and PD mouse models, SNc DaNs are more prone to neuron death compared to their counterparts in the neighboring ventral tegmental area (VTA). This pattern of selective neurodegeneration can also be observed in the differential loss of dopaminergic projections in the striatum (Bregma 0.74 mm, $20\text{ }\mu\text{m}$ sections, 1 mm scale bar), where the dorsal part, the caudate putamen (CPu), which is primarily innervated by SNc DaNs, is more vulnerable than the ventral part, the nucleus accumbens (Acb), which contains dopaminergic projections originating in the VTA

3.2 Immuno-histochemistry of Free-Floating Brain Sections

1. Wash the sections in $1 \times$ PBS for 10 min on a shaker to remove traces of NaN_3 .
2. Incubate the sections in 0.9% H_2O_2 for 10 min on a shaker to quench endogenous peroxidases (*see Note 12*).
3. Wash the sections 5×5 min in $1 \times$ PBS to remove traces of H_2O_2 (*see Note 13*).
4. Incubate the sections in blocking serum (normal serum) provided by the VECTASTAIN[®] Elite ABC Kit Peroxidase kit (150 μL of stock in 10 mL $1 \times$ PBS) for 1 h (Alternatively 5% normal goat serum in $1 \times$ PBS can be used. The species of the serum for the blocking solution should be the same as the source of the secondary antibody).
5. Incubate the sections in the primary antibody overnight diluted in blocking buffer at 4 °C on a shaker (*see Note 14*). For stereological quantification of SNc DaNs, we commonly use a tyrosine hydroxylase (TH) antibody (polyclonal rabbit, Sigma-Aldrich, #ab152, 1:5000, *see Note 15*). Seal the plate using PARAFILM[®] to avoid dehydration of the sections.
6. Wash the sections 5×5 min in $1 \times$ PBS.
7. Incubate the sections in biotinylated secondary antibody provided by the ABC kit (50 μL of biotinylated secondary antibody stock in blocking serum [normal serum, 150 μL of stock in 10 mL $1 \times$ PBS, *see step 4*]) for 1 h on a shaker.
8. Wash the sections 5×5 min in $1 \times$ PBS.
9. Prepare the ABC solution 15–30 min before usage: Add 100 μL of Reagent A and 100 μL of Reagent B to 5 mL of $1 \times$ PBS and mix immediately.
10. Incubate the sections in the ABC solution for 1 h on a shaker.
11. Wash the sections 5×5 min in $1 \times$ PBS.
12. Prepare the DAB solution: Add 2 drops ($\sim 84 \mu\text{L}$) of DAB reagent 1, 2 drops of DAB reagent 3 and 4 drops ($\sim 100 \mu\text{L}$) of DAB reagent 2–5 mL of water. Two drops ($\sim 84 \mu\text{L}$) of Nickel Solution can also be added to produce a gray-black reaction product. Mix well before use.
13. Incubate the sections in the DAB solution. Observe the staining process carefully (useful to do over a white surface) to avoid saturation of the staining. Typical exposure times range from 2 to 10 min (*see Notes 16 and 17*).
14. Wash the sections 3×5 min in $1 \times$ PBS.
15. Carefully mount the sections on Superfrost plus slides in a logical order (e.g., from rostral to caudal) using a brush in a small container with water and let them dry for ~ 1 h (alternatively the slides can be dried overnight).

16. Dehydrate the sections in increasing ethanol solutions (1 min in 50%, 2 min in 70%, 2×2 min in 95%, and 1 min in 100%) and clear 5 min in xylenes.
17. Coverslip the sections in Permount™ and dry for at least 1 h. Continue to microscopy as soon as the slides are dried. Stained sections can be stored at RT (ideally light-protected) for an indefinite time.

3.3 Microscopy and Stereological Quantification Using the Optical Fractionator

Unbiased stereological quantification of SNc DaNs using the optical fractionator approach is performed as described before [14, 20, 21]: For each brain, a total of eight coronal sections at an interval of 240 μ m (every sixth serial section) are used within the cranial and caudal limits of the SNc (Bregma -2.54 to -3.88 mm [23], see **Note 18**). Below, we summarize the steps to be performed using an upright brightfield microscope equipped with a motorized specimen stage (Leica MICROSYSTEMS DMRXE microscope) and a computer with Stereo Investigator® software (version 11.06.2):

1. Place the stained sections in the motorized specimen stage of the microscope.
2. Turn on the microscope, the computer and switch to the 10 $\times$ objective at the microscope.
3. Start the Stereo Investigator® software and set the microscope to view the image on the computer screen.
4. For white balancing, find a blank area on the slide and select *Adjust Video Input > White Balance > Automatic > OK*.
5. Select *Tools > Serial Section Manager > New Section* and label each section: *Section Name*, *Number of Sections Total* (8), *Evaluation Interval* (240 μ m), *Section Cut Thickness* (40 μ m), *Mounted Thickness* (Calculate the thickness every tenth frame. In our hands processing of 40 μ m sections typically result in a thickness of 12–20 μ m) and *Starting Z level* (0).
6. Select *Check Probes > Define Counting Frame* and set to 75 μ m $\times$ 75 μ m. Select *MacroView* and *Delineator Contour Name* from the dropdown box (If you aim to count multiple markers you can identify them here).
7. Select *10 $\times$ Objective* and manually outline the region of interest, the SNc (see Fig. 1), by drawing a contour line around it. Do not include regions not intended to be analyzed (Use the *Eraser* function if needed). Select *Close Contour* when you are nearly done outlining the SNc to connect the ends of your line. At this point, multiple regions of interests can be outlined, depending on the experimental setup (see **Notes 19** and **20**).
8. Repeat for all sections.

9. Select *100× Objective* (Switch to 100× objective at the microscope) and adjust the condenser, light intensity, and focus as required to locate your sample.
10. Select the appropriate marker for counting.
11. Select *Probes > Optical Fractionator* and click the first contour.
12. Set the software to the following settings: *XY Placement Counting Frames*: 150 μm $\times$ 150 μm , *Dissector Probe Setting*: fixed distance 1 μm guard, *Dissector Optical Height*: 20.00 μm .
13. Sampling of counting frames will be performed randomly. For each sample site, scroll through the *z*-axis and select *Ok* if marked neurons are present. Left click to label focused neurons with the desired marker if they are located within the counting frame or touching the open frame (green line), but not touching the forbidden frame (red line). At this point multiple markers can be used to quantify different cell types (see **Notes 14** and **20**).
14. Continue for all counting frames.
15. Select *Display Probe Run List*. Select your row of interest. Select *View Results* (Select desired marker if using multiple markers): Lines will report the neuron number per section and can be added together for the total number of SNc DaNs (*N*) per brain (see **Note 21**).
16. Transfer the data for further analysis to spreadsheet and statistical analysis software (e.g., Microsoft Excel and GraphPad Prism, see **Note 22**).

The following descriptions outline the quantification of SNc DaNs numbers using the physical fractionator approach following IHC performed with 5 μm paraffin sections. In principle, perfusion, brain dissection, and immunohistochemistry can be done as suggested above (all protocol deviations are described below).

3.4 Tissue Processing and Paraffin Sections

1. Following the dissection of the brain and its incubation in 4% PFA at 4 °C for at least 24 h, dehydrate the brain in a series of ethanol solutions of increasing concentrations (15 min in 70%, 15 min in 90%, 2 $\times$ 15 min in 100%, 30 min in 100%, and 45 min in 100%) using a tissue processor (Leica MICROSYS-TEMS ASP300).
2. Embed dehydrated tissue in liquid paraffin using an embedding station (Leica MICROSYS-TEMS EG1150H): Place the tissue in the designated paraffin mold and fill the mold with molten paraffin. Hold the brain using blunt forceps in a fixed position with its cranial end (olfactory bulb) facing the ground of the form until the paraffin has cured (paraffin turns white).
3. Keep the mold on a cold plate (4 °C) for at least 1 h. Remove the paraffin block with the brain from the mold.

4. Trim the paraffin block by removing paraffin around the brain using a sharp knife (maintain a paraffin block around the brain of $\sim 3\text{ cm} \times 3\text{ cm}$).
5. Mount the paraffin block in the cassette clamp of the microtome (Leica MICROSYSYSTEMS RM2125 RTS), the lateral side of the brain facing the knife and the dorsal side facing to the left, and section the brain at a thickness of $5\text{ }\mu\text{m}$. In this orientation coronal sections from cranial to caudal will be produced. Try to avoid the loss of cut material. Sectioning should be performed with a clean, sharp blade. Sectioning is simplified by frequently breathing on the cutting surface.
6. Using a fine brush or forceps, carefully transfer the sections to a heated water bath ($40\text{--}50\text{ }^{\circ}\text{C}$, $5\text{--}10\text{ }^{\circ}\text{C}$ below the melting point of paraffin, Leica MICROSYSYSTEMS HI1210) to unfold the sections.
7. Transfer the brain sections to a slide (3 sections per slide). Maintain the order of sections produced from cranial to caudal and label slides accordingly. Brains in paraffin blocks and paraffin sections in a slide box can be stored at RT for indefinite time.

3.5 Immunohistochemistry of Paraffin Brain Sections

1. Deparaffinize sections $3 \times 5\text{ min}$ in xylenes.
2. Rehydrate sections in decreasing ethanol solutions (v/v% in water: $2 \times 5\text{ min}$ in 100%, 5 min in 70%, 5 min in 50%).
3. Wash sections 5 min in $1 \times \text{PBS}$.
4. Antigen retrieval: In a water bath, heat sodium citrate buffer to $95\text{ }^{\circ}\text{C}$ in a glass beaker and immerse slides in hot buffer for 20 min using a slide holder.
5. Wash sections $5 \times 5\text{ min}$ in $1 \times \text{PBS}$.
6. Dry tissue-free edges of slides and circle tissue with Super PAP Pen.
7. Continue with **step 2** of Subheading 3.2 (above, ignoring **step 15**).

3.6 Microscopy and Stereological Quantification Using the Physical Fractionator

Unbiased stereological quantification of SNc DaNs using the physical fractionator approach is performed as described before [22]: For each brain, a total of 9 pairs (two adjacent sections) of coronal sections at an interval of 21 serial sections (every seventh slide) are used within the cranial and caudal limits of the SNc (Bregma -2.54 to -3.88 mm [23], *see Note 18*). According to the principle of the physical fractionator, neurons are identified in two adjacent thin ($5\text{ }\mu\text{m}$) sections and are only counted if their profile appears in the reference section (section a) but not in the lookup section (section b) [24]. For this quantification approach, microscopy of stained

pairs of sections can be performed with a brightfield microscope of choice. However, we recommend using an automated microscopy system (Leica MICROSYSTEMS SCN400 slide scanner):

1. After having performed white balancing and having focused on neurons of the midbrain, scan the midbrain of every stained section using a $40\times$ objective.
2. Transfer the scan to a slide viewing software (Leica MICROSYSTEMS SlidePath Gateway), identify the SNc and take a first picture (1) of that micrograph at $11\times$ magnification. Rename the first picture according to animal-ID, slide-ID and add “-1” to the name.
3. Slowly move the scan vertically to a neighboring region and take a second picture (2) at the same magnification. Make sure that the peripheries of both pictures (1 and 2) overlap to $\sim 25\%$ (required for composite picture). Rename the second picture accordingly.
4. Continue for the whole midbrain region in the scan (both hemispheres) and repeat for scans of all stained sections.
5. Transfer all pictures of a scan to the image stitching software (Microsoft Image Composite Editor), stitch the pictures to a single composite picture and export the composite picture as Tag Image File Format (TIFF).
6. Rename the composite picture according to animal-ID, slide-ID, and section-ID (section “-a” for reference and “-b” and for lookup section). Repeat for all scans.
7. Transfer the composite pictures a and b of 2 adjacent sections to the image editing software (Adobe Photoshop or GNU Image Manipulation Program) as two separate layers (layer a and b). Do not change the scale of the pictures.
8. Set the opacity of the top lookup layer to $\sim 50\%$ and rotate the pictures until they properly overlay (use the tissue edges and the stained cells as landmarks).
9. Add the physical fractionator sampling grid (area of sampling grid = $191.82\ \mu\text{m}^2$, area of counting frame = $42.9\ \mu\text{m}^2$) as an additional layer and randomly rotate the grid.
10. Zoom into the first counting frame of choice, covering parts of the stained SNc, hide the lookup section layer (Press the “eye” icon in the *Layers* window) and label all neurons in the reference section layer in a colour of choice (e.g., red), using the *Pencil Tool*. Label neurons only if they are located within the counting frame or touching the open frame (dashed line), but not touching the forbidden frame (solid line).
11. Show the lookup section layer and, using a second color (e.g., yellow), label all neurons of the same counting frame in the lookup section layer (while the reference section is hidden).

Show both layers and count all labeled neurons which only appear in the reference section (neurons which are only labeled red). Note the neuron number and continue for all counting frames covering the stained SNc.

12. Repeat for all section pairs. Do not change the rotation of the grid during the counting process.
13. Calculate the sum of all labeled SNc neurons (Q) for the total number of SNc DaNs (N). The *height sampling fraction* ($hsf = \text{section thickness/dissection height} = 5 \mu\text{m}/5 \mu\text{m} = 1$), the *area sampling fraction* ($asf = \text{area of counting frame/area of sampling grid} = 42.9 \mu\text{m}^2/191.82 \mu\text{m}^2 = 0.223647$) and the *section sampling fraction* ($ssf = 1/\text{interval between analyzed section pairs} = 1/21$) are given as constants. The total number of SNc DaNs (N) is calculated by means of the following equation [25].

$$N = Q \times \frac{1}{hsf} \times \frac{1}{asf} \times \frac{1}{ssf} = Q \times \frac{1}{0.223647} \times \frac{1}{0.047619}$$

14. Transfer the data for further analysis to spreadsheet and statistical analysis software (e.g., Microsoft Excel and GraphPad Prism).

3.7 Optical Densitometry in the Striatum

Analysis of the TH signal intensity in the striatum stained with the protocols described above (using either free-floating cryosections or paraffin sections) can be performed to examine the loss of dopaminergic projections originating in midbrain nuclei (*see Notes 19 and 23*). Typically, TH staining is performed at Bregma 0.74 mm (*see Fig. 1*). Striatal densitometry can be performed using micrographs obtained with a brightfield microscope of choice. We recommend using an automated microscopy system, enabling to scan the whole brain section (as described in a) but analysis can also be performed in a partial micrograph of the striatum (b):

1. Transfer (drag and drop) the micrograph as a JPEG (RGB color image) to NIH ImageJ.
2. Transform the micrograph into an 8-bit grayscale image: Select *Image > Type > 8 bit*.
3. Invert the image: Select *Edit > Invert*.
4. Select *Analyze > Select Measurements > Integrated density (a) or Mean gray value (b)*.
5. Outline the striatum (*see Note 19*). (a) Using the “Polygon selections” tool, outline the striatum in both hemispheres and the *corpus callosum* as a background reference.
(b) Using the “Rectangle” tool, outline the region of interest in the striatum. Select *Analyze > Tools > ROI Manager > Add* to add the outlined region of interest to the ROI Manager and select *More > Save* to save the outline.

6. Label a second and third region of interest in the same micrograph using the saved outline (all three regions of interest have the same area).
7. Repeat in a micrograph of the corpus callosum of the same section.
8. Select *Analyze > Measure* and transfer the values for signal intensities (integrated density or mean gray value) to Microsoft Excel.
9. Average the signal intensities of each brain region per section (for a and b: the intensities of both hemispheres, for b: the intensities of each region of interest) and subtract the signal intensity of the corpus callosum from the striatal signal intensity.
10. Repeat for all images of the technical replicates per group (ideally three images per biological sample).
11. Transfer the data for further analysis to statistical analysis software (e.g., GraphPad Prism).

4 Notes

1. The following steps should be performed in a ventilated hood: production of 4% PFA, perfusion of the mice, freezing of the brains in isopentane, incubation of the sections with DAB solution, clearing/deparaffinization of the sections with xylenes and mounting of the sections with Permount™.
2. We recommend perfusing (and staining) as many replicates as possible of all experimental groups simultaneously. Differences in the perfusion quality may result in different staining intensities.
3. At this point a sample (e.g., ear punch or tail tip) can be collected to confirm the genotype of the mouse. This should be done before the perfusion with PFA.
4. Avoid blocking the carotid arteries by applying too much pressure on the neck.
5. Butterfly needles can be pinned to the Styrofoam block, providing more stability than conventional needles. Alternatively to using syringes, the intracardiac perfusion can be performed using gravitational force (with the solution reservoirs hanging approximately 50 cm above the mouse during the perfusion) or with a peristaltic pump set to 1–2 mL/min.
6. 10% buffered formalin can be used instead of 4% PFA to fix the mouse body.

7. Researchers who lack detailed knowledge about the mouse brain anatomy should familiarize themselves with the brain region of interest using a mouse brain atlas [23] before brain dissection and sectioning.
8. At this stage, the brain can be engulfed in O.C.T. on dry ice using disposable molds. Fill the mold to 1/3 with O.C.T., quickly place the brain with its caudal side in the O.C.T. in a fixed position using prechilled, blunt forceps and completely fill the mold with O.C.T. O.C.T. blocks can be stored at -80°C for several years.
9. Stereology requires serially cut sections without loss of any brain material in the region of interest. Also, sections must be symmetrical for stereology which primarily depends on the orientation of the brain toward the blade (the angle at which the sections are produced). We recommend practicing sectioning using brains of relative low value (e.g., control brains) before processing experimental tissue. Consistent labelling of the section order and the specimen during sectioning helps to avoid confusion. Loss of midbrain sections should be recorded for the precise documentation of the interval between analyzed sections. Brains where an excessive amount of material was lost during sectioning should be excluded from stereological analysis.
10. If the lesion is only induced in the SNc of one hemisphere, neuron counts can be compared between the lesioned hemisphere and the nonlesioned, contralateral hemisphere as a control [26]. In this situation, it is *crucial* to identify the lesioned/treated side at an early stage. In our hands, this is done while sectioning: a small hole is introduced to the side of the brain that is untreated at a site that is not analyzed downstream (e.g., thalamus or cortex). In this way, should the section flip while in suspension, the investigator can be confident in the region treated.
11. Refill the $1\times$ PBS + 0.02% NaN_3 solution in the wells of the plate containing the brain sections at 4°C every 3–6 months to avoid dehydration of the brain sections.
12. Incubations during the staining procedure can be performed in a 12-well plate, using as little as 500 μL per well.
13. Free-floating sections can easily and safely be transferred between wash solutions ($1\times$ PBS) in well plates using net bottom wells.
14. False-positive signals by direct, unspecific target protein-secondary ab interactions can be examined by performing the staining procedure with and without primary ab in parallel on separate sections (ideally on the same slide if possible) and consecutive comparison of signal intensities. A high difference

of signals of sections stained with and without primary ab indicates that the signal results from a specific interaction between the target protein and the primary ab and not by an unspecific interaction between target protein and secondary ab.

15. Assessment of the dopaminergic system is commonly achieved by using standard DaN protein markers, such as TH or dopamine transporter (DAT) [27]. Reduced activity of DaNs before cell death may be accompanied by the reversible reduction of TH expression, a state which has been described as “dormancy” [28, 29]. To avoid misinterpretation of reduced TH signal intensity as loss of DaNs numbers, we recommend using multiple DaNs markers, panneuronal markers (e.g., NeuN) and/or pancellular markers (e.g., cresyl violet or Nissl staining) for stereological quantification [14, 30, 31].
16. The most important step to be consistent in the staining process is the exposure to DAB: All sections needed for the experimental comparison must be exposed to DAB for the same time, as differences in the DAB incubation time result in different staining intensities which can impact the analysis (especially for optical densitometry). Always stain similar amounts of replicates of the experimental group and its control group simultaneously.
17. For new antibodies or other tissue types, the optimal DAB exposure duration should be tested on few sections before proceeding with the batch analysis.
18. For scientists who are inexperienced in brain anatomy it may be challenging to identify the dopaminergic midbrain nuclei in mouse brain sections. Although only 8–10 stained sections are needed to quantify SNc DaNs using stereology, a pilot staining for a DaN marker on sections with uniform spacing (e.g., every tenth section) of the whole brain may be performed, as a reference for further analysis.
19. In PD, SNc DaNs and their striatal projections are more affected by neuron death than VTA DaNs [2, 3, 5, 6, 8]. To test if this differential vulnerability is reflected in a PD mouse model, integrity of the dopaminergic system should be investigated in both, the SNc, as well as in the VTA, but also in their respective striatal projection areas (*see* Fig. 1).
20. Stereology is not restricted for the quantification of a specific cell type (here DaNs) in a certain organ (here the SNc) and can in principle be applied to any kind of detectable particle in a physical object. Of note, stereological quantification is commonly applied to the assessment of different neuron types [32, 33], nonneuronal cells [34, 35], and other particles such as Lewy bodies or Lewy neurites [36].

21. The total amount of SNc DaNs estimated by stereology depends on the tissue processing and staining procedures, the quantification approach (the fractionator type) and can vary between the persons conducting the quantification. In our hands, counting DaNs (TH-positive neurons) using stereology in the SNc of mice typically results in total neuron numbers of 10,000–12,000 DaNs [14, 20–22].
22. Neurodegeneration of animal models is commonly described as a significant reduction of neuron numbers in the experimental PD group (KO-, transgenic, toxin-treated animals, etc.) compared to its control group (WT, vehicle-treated animals, etc.) in the postlesional phase (elevated age, post-toxin treatment, etc.), as opposed to no differences in neuron numbers between groups before exposure to the insulting stimulus. Statistical analysis with two variables (e.g., genotype and time) and four groups should be performed with two-way analysis of variance (ANOVA). Post hoc comparisons between individual groups should only be performed if at least one of the main factors (e.g., genotype or time) or the interaction of the ANOVA reaches significance.
23. Loss of dopaminergic terminals in the striatum precedes degeneration of SNc DaNs, a phenomenon which is referred to as “dying back” process [4]. We recommend analyzing the optical densitometry of dopaminergic projections in the striatum before the time- and labor-intensive assessment of SNc DaNs by stereology, especially when describing a novel PD model where nigrostriatal degeneration has not been investigated yet.

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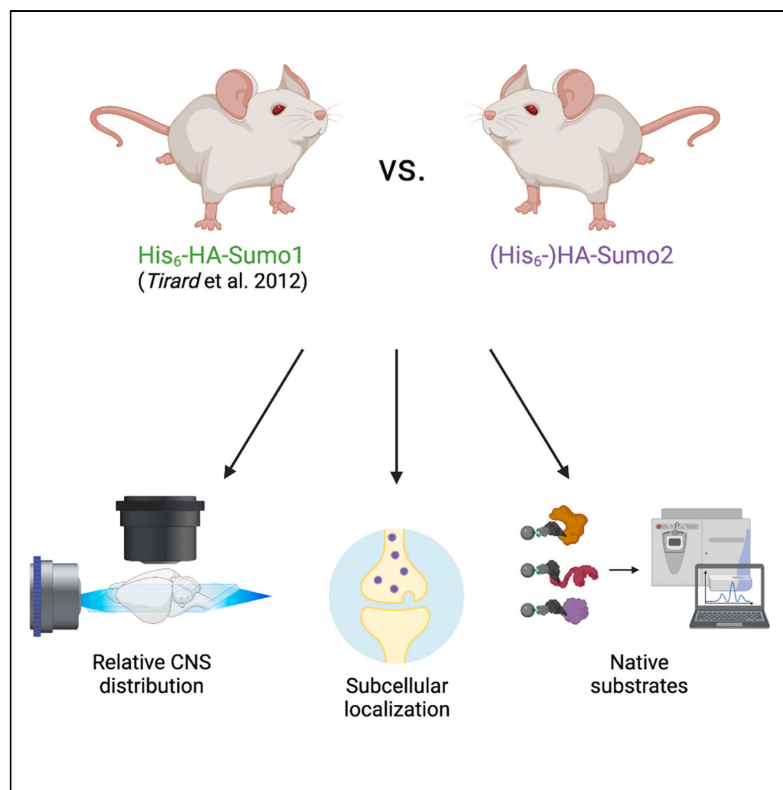
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Contributions: I helped perform animal handling, tissue processing, and tissue staining. My overall contribution to the paper was about 10%.

Article

Characterizing the differential distribution and targets of Sumo1 and Sumo2 in the mouse brain



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Highlights

(His₆-)HA-Sumo2 knockin mice enable direct comparison of Sumo paralogs *in vivo*

Whole-brain imaging reveals regional differences between Sumo1 and Sumo2 expression

Sumo2 localizes to synapses *in vivo*

Sumo1 and Sumo2 have shared unique targets in the mouse brain

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Article

Characterizing the differential distribution and targets of Sumo1 and Sumo2 in the mouse brain

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SUMMARY

SUMOylation is an evolutionarily conserved eukaryotic posttranslational protein modification with broad biological relevance. Differentiating between the major small ubiquitin-like modifier (SUMO) paralogs and uncovering paralog-specific functions *in vivo* has long been very difficult. To overcome this problem, we generated His₆-HA-Sumo2 and HA-Sumo2 knockin mouse lines, expanding upon our existing His₆-HA-Sumo1 mouse line, to establish a “toolbox” for Sumo1-Sumo2 comparisons *in vivo*. Leveraging the specificity of the HA epitope, we performed whole-brain imaging and uncovered regional differences between Sumo1 and Sumo2 expression. At the subcellular level, Sumo2 was specifically detected in extranuclear compartments, including synapses. Immunoprecipitation coupled with mass spectrometry identified shared and specific neuronal targets of Sumo1 and Sumo2. Target validation using proximity ligation assays provided further insight into the subcellular distribution of neuronal Sumo2-conjugates. The mouse models and associated datasets provide a powerful framework to determine the native SUMO “code” in cells of the central nervous system.

INTRODUCTION

Posttranslational modifications (PTMs) can alter the secondary, tertiary, and quaternary structure of proteins and thereby dynamically regulate their function. Although many PTMs involve the covalent conjugation of small moieties, such as phosphate, glycosyl, or acetyl groups, protein function is also modulated by reversible covalent conjugation of large molecules, including the protein ubiquitin and ubiquitin-like modifiers, such as NEDD8, or small ubiquitin-like modifiers (SUMOs).^{1–7}

Conjugation of SUMOs (“Sumo” in mice) through SUMOylation is a dynamic and essential process that has been conserved throughout eukaryote evolution.^{1,2} In its course, immature SUMOs are first matured by sentrin-specific protease (SENP) cleavage at a C-terminal diglycine motif that is essential for conjugation.³ Next, SUMOs are activated in an ATP-dependent manner and conjugated to the E1 ligase heterodimer consisting of SUMO-activating enzyme 1 (SAE1) and ubiquitin-like modifier activating enzyme 2 (UBA2).⁴ SUMOs are then transferred to the sole E2 ligase of the system, SUMO-conjugating enzyme UBC9 (UBC9, encoded by *UBE2I*), which is essential for SUMOylation.^{5–10} SUMOylation occurs at lysine residues of target substrates, typically in SUMO consensus motifs (Ψ-K-x-D/E; Ψ, large hydrophobic), either solely via UBC9 or upon facilitation by E3 ligases.^{11,12} SENPs readily deSUMOylate substrates, thus recycling SUMOs for subsequent conjugation rounds and allowing for highly dynamic substrate regulation.¹³

There are five human SUMO paralogs (SUMO1–SUMO5), of which SUMO1–SUMO3 have been studied most extensively as they are ubiquitously expressed and found in most vertebrates, whereas SUMO4 and SUMO5 are specific to humans and exhibit tissue-specific expression.^{14,15} SUMO1 is ~50% homologous to SUMO2 and SUMO3, whereas SUMO2 and SUMO3 are 97% homologous and often referred to together as SUMO2/3.¹⁶ While SUMO paralogs have multiple redundant roles and can mutually compensate paralog loss in several contexts, each paralog also plays unique roles, e.g. by localizing to different distinct subcellular regions and by targeting different substrates.^{2,14} In addition, SUMO2, but not

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SUMO1 or SUMO3, is the only essential paralog so that its knockout in mice causes major developmental defects and embryonic lethality by E10.5.¹⁷ The essentiality of SUMO2 for proper development may be in part due to higher levels of SUMO2 in comparison to its paralogs,^{17,18} but is likely also a result of a specific subset of SUMO2 substrates in cells. Thus, characterizing the specific roles of SUMO2 is crucial to uncover the key molecular and cellular processes that define SUMO biology and the mechanisms by which SUMOylation affects development and disease processes such as neurodegeneration or the pathological consequences of ischemic stroke.^{19–23}

Several key challenges have limited studies in SUMO biology: (i) Differentiating between SUMO paralogs using antibodies is very difficult or even impossible due to the high degree of homology²⁴; (ii) the highly dynamic nature of SUMOylation requires consideration of biological context, e.g. tissue, cell type, or cell state, for capturing and characterizing SUMOylation events, particularly *in vivo*; (iii) many tools and model systems used to study SUMOylation are limited, particularly when they rely on overexpression approaches, where off-target SUMOylation is a major potential confound. Consequently, protein SUMOylation has chiefly been studied in highly proliferating cells, where SUMOylation is now well known to control multiple nuclear processes, such as DNA repair or transcriptional control.^{19,25} In contrast, the function of SUMOylation in extranuclear compartments, post-mitotic cells, or complex tissues is far less well understood. The role of extranuclear SUMOylation in post-mitotic neurons is a prime example in this regard, with major controversies surrounding evidence supporting^{26–31} and opposing^{32–34} a key role of SUMO1 conjugation in synaptic compartments.

Our strategy to overcome some of the technical limitations in studying SUMO biology has been to generate knockin mouse models that express endogenously tagged Sumo, allowing for stringent approaches that leverage the specificity and affinity of epitope tags to identify Sumo-targets and characterize their subcellular localization, using wild-type littermates as negative controls and flanked by studies on Sumo knockout mice. The first allele we generated—a His₆-HA-Sumo1 mouse—overcame what we viewed as technical limitations of previous studies on the role of Sumo1 in neurons, and surprisingly did not provide evidence for the localization of Sumo1 at synapses.^{33,34} While the His₆-HA-Sumo1 model facilitates the study of Sumo1 *in vivo*, expanding the toolkit to other Sumo paralogs will provide powerful opportunities to uncover the complexities of Sumo-mediated regulation.

We have now expanded our mouse genetic tool-generation efforts in SUMO biology to promote further studies on the role of SUMOylation *in vivo* and in complex cellular systems such as neurons. Specifically, we generated and characterized in parallel two novel knockin mouse models that express either an HA-Sumo2 or a His₆-HA-Sumo2 fusion protein from the endogenous *Sumo2* locus to characterize Sumo2 and its substrates *in vivo*. Analogous to our work with the corresponding His₆-HA-Sumo1 mouse,^{32–34} these knockin mouse lines allow to directly compare the localization and conjugation patterns of Sumo1 and Sumo2, which we did for the central nervous system (CNS). We used whole-brain imaging to map the relative abundance of Sumo1 and Sumo2 in the adult mouse brain and observed that both are broadly distributed in the CNS but exhibit defined regional differences. Importantly, we found that Sumo2, unlike Sumo1,^{32–34} is present in both nuclear and extranuclear compartments, the latter including neuronal synapses. Given this distinct subcellular distribution, we explored native Sumo1 and Sumo2 targets using anti-tag immunoaffinity purification from knockin mice and mass spectrometry. We identified genuine Sumo2-specific targets in the adult mouse brain, including a subset of non-nuclear proteins. Moreover, we observed dynamic changes between Sumo1- and Sumo2-selective substrates. Finally, we validated several hits using proximity ligations assays to detect protein-Sumo interactions in cultured cortical neurons. This approach did not only provide an additional mode of validating native Sumo2 interactors in wild-type neurons but also yielded spatial information, again demonstrating both nuclear and cytoplasmic interactions between Sumo2 and its substrates. Together, the two knockin mouse lines and our data provide an important resource that lays the foundation of a “SUMO-code” as an additional layer of molecular complexity in brain function.

RESULTS

Generation of (His₆)-HA-Sumo2 mouse lines

The use of epitope tags provides a versatile way to streamline the study of proteins by taking advantage of the highly selective nature of antibodies raised against these tags. In parallel institutions, we generated two independent alleles to facilitate the study of Sumo2 *in vivo*. We took advantage of a CRISPR/Cas9 approach



to knockin a 6xHis-hemagglutinin (*His₆-HA*)-tag into the amino-terminus of *Sumo2* (*His₆-HA-Sumo2*) in C57BL/6J mice (Figure S1A) in one case, mirroring that of a previously generated mouse line to study *Sumo1* *in vivo*.³² Moreover, we also generated an *HA-Sumo2* knockin mouse that does not have the *His₆* tag. We observed that knockin of either *His₆-HA* or *HA* to *Sumo2* resulted in hydrocephaly and premature death in heterozygous mice, indicating a likely hypomorphic effect of the epitope tag on a C57BL/6J background. Interestingly, backcrossing the mice to an FVB/N background alleviated these effects, resulting in no overt differences between mutant and wild-type FVB/N (WT) littermates in either line. Nevertheless, given the importance of *Sumo2* for life, and the robustness of the *HA*-tag for visualization and biochemistry, we performed the bulk of our analyses on heterozygous *His₆-HA-Sumo2* (and heterozygous *His₆-HA-Sumo1*; also crossed to an FVB/N background) knockin mice.

***His₆-HA-Sumo2* enables the comparison of endogenous *Sumo2* levels in the CNS**

To determine whether the addition of the *HA* epitope tag affects native *Sumo* levels, we assessed the levels of *Sumo2/3* conjugates by Western blot, comparing total levels of unconjugated *Sumo* ("Free *Sumo*") and *Sumo*-conjugated proteins from mouse brain lysates. We observed no change in the abundance of *Sumo2/3* conjugates in the brains of adult *His₆-HA-Sumo2* mice compared to *His₆-HA-Sumo1* or WT mice (Figure S1B). As decreased levels of *Sumo1* conjugates were previously observed in homozygotes *His₆-HA-Sumo1* mouse brain,³³ we thus measured whether there were any changes in the abundance of native *Sumo1* conjugates in heterozygous *His₆-HA-Sumo1* and *His₆-HA-Sumo2* mice.¹⁷ Again, no changes were observed between *Sumo* knockin line and their WT counterparts, indicating that the heterozygous *His₆-HA-Sumo2* or *His₆-HA-Sumo1* allele does not alter overall levels of *Sumo* conjugates in mouse brain (Figure S1C). In addition, we did not observe a significant effect on the expression of *Sumo1*, *Sumo2*, or *Sumo3* transcript levels in either of the *His₆-HA-Sumo* knockin mice (Figure S1D). Thus, the novel *His₆-HA-Sumo2* mouse model enables the study of *Sumo2* at endogenous levels *in vivo* without changes to the *Sumo* equilibrium.

The uniformity of the *HA*-tag epitope between the *His₆-HA-Sumo1* and *His₆-HA-Sumo2* mice allows for a direct comparison of endogenous *Sumo1* and *Sumo2* levels *in vivo*. We assessed the relative levels of *Sumo* expression in the CNS, specifically the cortex (CTX), cerebellum (CBM), olfactory bulbs (OB), spinal cord (SC), and remainder of the brain (termed "striatum-thalamus-brainstem" or "S-Th-B" for simplicity) from adult mice (Figure 1A). Real-time qPCR analysis of WT mice shows that *Sumo2* is the *Sumo* paralog most abundantly expressed in all regions of the CNS (Figure S1E). In addition, *Sumo2* expression varies significantly between CNS regions, whereas *Sumo1* and *Sumo3* exhibit more uniform expression (Figure 1B). Western blot analysis from CNS regional lysates of *His₆-HA-Sumo1*, *His₆-HA-Sumo2*, and WT mice shows that levels of conjugated and free *His₆-HA-Sumo1* indeed do not significantly vary between CNS regions, whereas free *His₆-HA-Sumo2* levels reflect the higher expression and variations observed at the RNA level (Figures 1C, S1F, and S1G). In addition, the OB is particularly enriched for high molecular weight *Sumo2*-conjugated proteins suggesting that *Sumo2* may play unique regional roles throughout the CNS (Figure S1F).

Regional analysis of *Sumo1* and *Sumo2* throughout the mouse brain

As differences in *Sumo* levels are detectable from regional crude brain lysates, we leveraged the specificity of the *HA*-tag and performed whole-brain clearing and mapping of *Sumo1* and *Sumo2* signals in the adult mouse brain. *His₆-HA-Sumo1* and *His₆-HA-Sumo2* mouse brains were cleared and immunolabeled against the *HA*-tag and NeuN before imaging using light sheet microscopy (Figure 2A, Video S1 and Video S2). Images were then analyzed and aligned to the Allen Brain Atlas to determine the relative levels of the *His₆-HA-Sumo* signals in defined brain regions (Table S1). It is worth noting that due to the different absolute abundance of *Sumo1* (low) versus *Sumo2* (high) in the brain, only qualitative *Sumo1* vs *Sumo2* comparisons were made. To generate relative maps of *HA-Sumo* distribution, the mean regional *HA* signal intensity was first normalized to the regional density of NeuN to account for changes in cell density before being averaged across hemibrains to account for tissue variability. Generally, *Sumo1* and *Sumo2* showed similar distributions throughout the mouse brain (Figures 2B–2E), and we observed that both *Sumo1* and *Sumo2* were most abundant in cerebellar nuclei.

While *Sumo* levels were generally evenly distributed when characterizing gross anatomical regions, clear nuances in *Sumo* abundance were detectable at higher anatomical resolution. For example, in the hippocampus, *Sumo* levels were highest in the parasubiculum and presubiculum, and lowest in the *Fasciola*

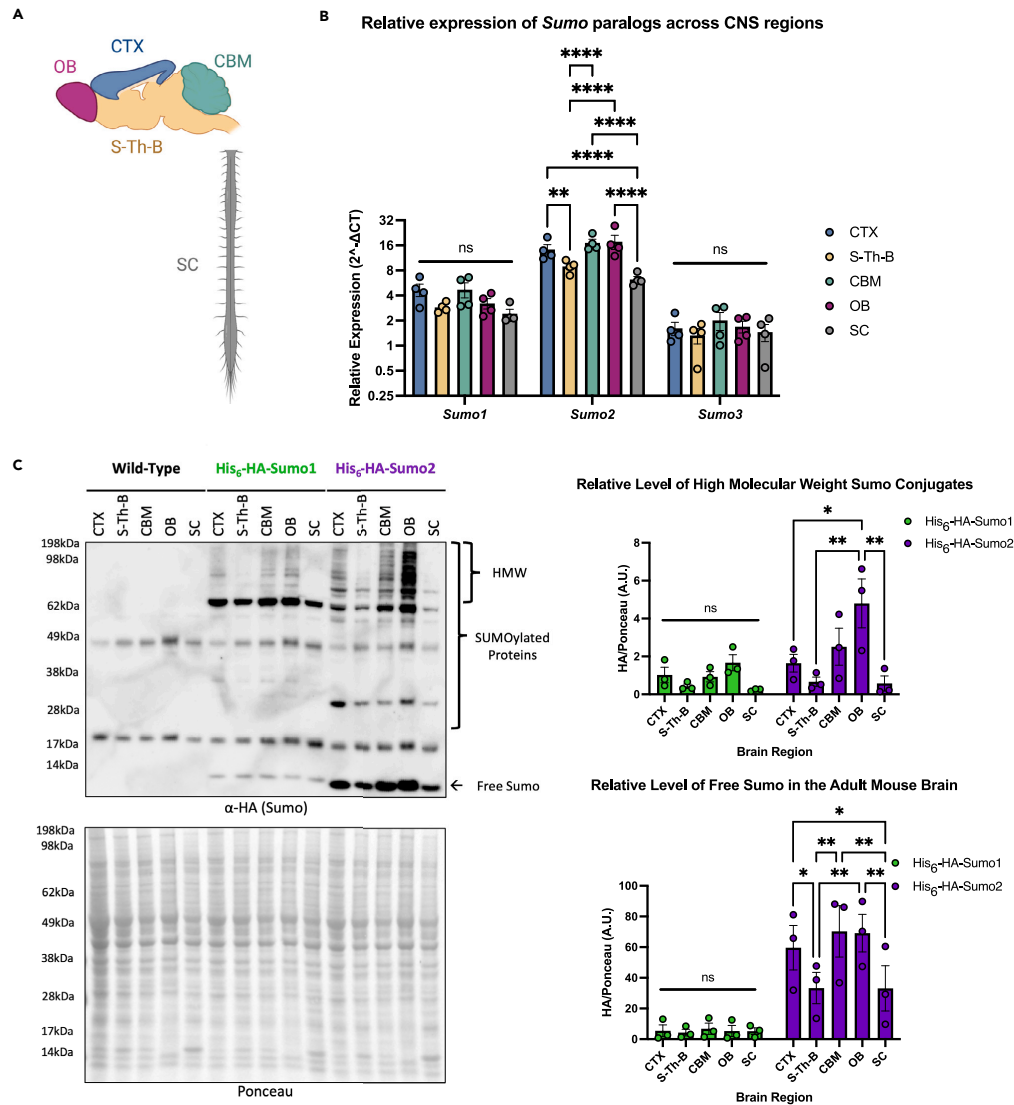


Figure 1. HA-epitope knockin enables direct comparisons of differential levels of Sumo1 and Sumo2 and their conjugates in the murine central nervous system

(A) Brain regions highlighted in the schematic were dissected for protein and RNA extraction and analyzed by Western blot using anti-HA antibody and RT-qPCR. CTX = cortex; S-Th-B = striatum-thalamus-brainstem; CBM = cerebellum; OB = olfactory bulb; SC = spinal cord.

(B) RT-qPCR analysis of WT mouse brain regions testing for significant regional changes for *Sumo1*, *Sumo2*, or *Sumo3* expression (N = 4).

(C) Western blot analysis of various regions of His₆-HA-Sumo1 and His₆-HA-Sumo2 mouse central nervous system. HA signal corresponding to free Sumo and SUMOylated proteins (HMW = High Molecular Weight) was normalized to ponceau and quantified via densitometry (N = 3). Two-way ANOVA tests with Tukey's post-hoc multiple comparisons analysis were performed. Data are presented as mean $\pm$ SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. See also Figure S1.

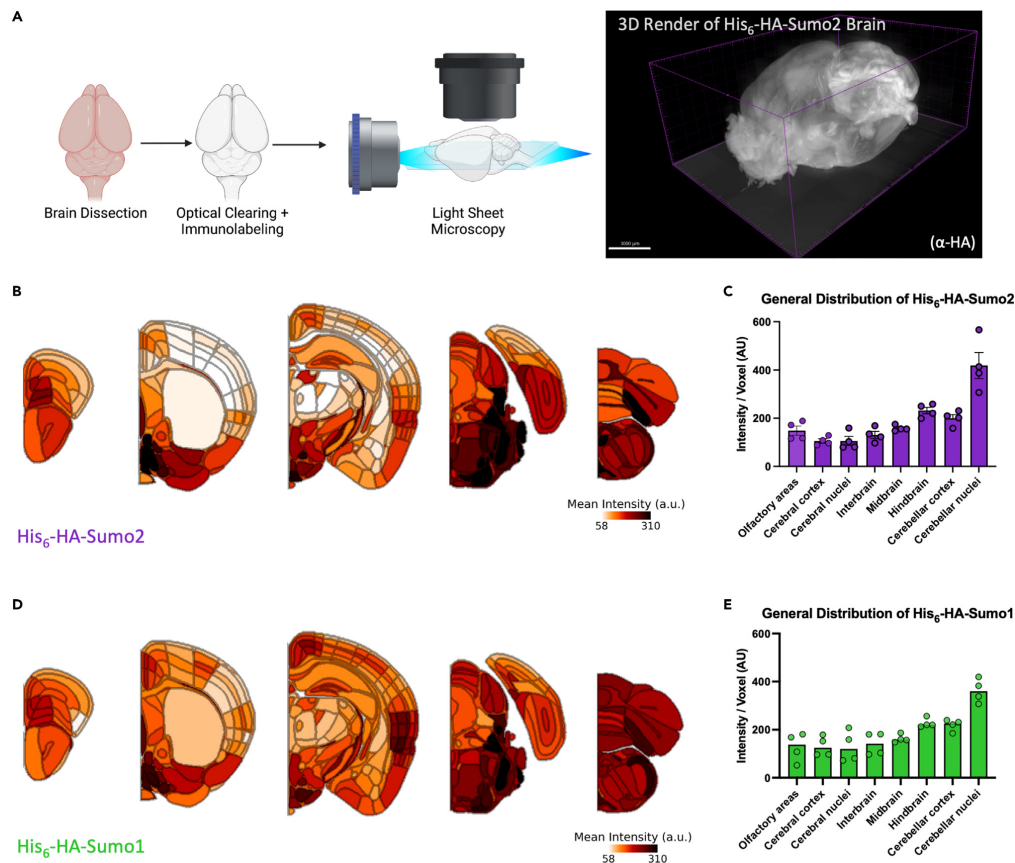


Figure 2. Whole-brain imaging reveals the topographical distribution of Sumo paralogs

(A) Schematic of brain clearing and light sheet microscopy for whole brain imaging with a 3D Imaris render of a His₆-HA-Sumo2 brain.

(B) Heatmaps of anti-HA immunosignal intensity normalized to regional density quantified by NeuN staining and alignment to the Allen Brain Atlas across the His₆-HA-Sumo2 depicted brain region, averaged between hemibrains and biological replicates (N = 2, two hemispheres/replicate).

(C) Bar plot depicting the relative levels of anti-HA immunosignal per voxel averaged across His₆-HA-Sumo2 brain regions defined by the Allen Brain Atlas.

(D) Heatmaps of anti-HA immunosignal intensity normalized to regional density quantified by NeuN staining and alignment to the Allen Brain Atlas across the His₆-HA-Sumo1 depicted brain region, averaged between hemibrains and biological replicates (N = 2, two hemispheres/replicate).

(E) Bar plot depicting the relative levels of anti-HA immunosignal intensity normalized to regional density across His₆-HA-Sumo1 brain regions defined by the Allen Brain Atlas. Each datapoint in (C) and (D) is from the mean intensity from a single hemisphere (N = 2, two hemispheres/replicate). Data are presented as mean ± SEM. See also Figures S2, S3, Table S1, Video S1, and Video S2.

cinerea (Figures S2A and S2B). In the neocortex, we observed that Sumo levels were generally enriched in layers 2/3 (Figures 2B, 2D and S2C), an area critical for integrative processing.³⁵ Interestingly, while Sumo1 and Sumo2 often share similar distributions, we observed that Sumo2 is enriched in the anterior hypothalamus (Figure S2D), particularly in regions involved in the circadian rhythm, including the suprachiasmatic nucleus and subparaventricular zone.³⁶

We investigated whether the brain-wide imaging findings could be validated by an orthogonal immunofluorescence assay (Figure S3). Looking at multiple brain regions, we found that Sumo1 and Sumo2 are broadly distributed through the somatosensory cortex. Interestingly, Sumo1 maintains a strictly nuclear

localization, whereas Sumo2 is observed throughout the nucleus and in the cytoplasm and axons of cortical neurons. In the cerebellum, we found that Sumo2 is specifically enriched in granule cells, contrasting the strong Purkinje cell staining observed in His₆-HA-Sumo1 mice.³² The hippocampus also displays characteristic nuclear staining for His₆-HA-Sumo1 in both the CA1 and CA3, whereas Sumo2 is seen throughout the nucleus and in the somata, and axons of hippocampal neurons. Thus, while Sumo1 and Sumo2 may share regional expression in the brain, the distinct localizations of Sumo1 and Sumo2 *in vivo* indicate that different targets of SUMOylation or different metabolism or conjugation dynamics of free Sumo likely play unique roles in neuronal regulation.

HA-Sumo2 localizes to synapses *in vivo*

Consistent with our previous work, specific Sumo1 immunosignal was mainly observed in neuronal nuclei (Figure S3).³² However, we noticed that Sumo2 signal can also be found outside the nuclear compartment (Figure S3). Thus, we further characterized extranuclear Sumo2 signals using homozygous HA-Sumo2 mice. As in the His₆-HA-Sumo2 model, levels of Sumo2 conjugates were unaltered in total brain lysates from homozygous HA-Sumo2 mice as compared to WT littermates, ruling out an effect of the HA-tag on overall Sumo2 expression and conjugation levels (Figure S4A).³² Western blot analysis of brain subcellular fractions using an anti-HA antibody highlighted not only the difference in global levels of Sumo1 and Sumo2 conjugates but also revealed that Sumo2 conjugates are enriched in non-nuclear fractions compared to their Sumo1 counterpart, including synaptic cytosol fractions S2 and S3 (Figures S4B and S4C). A weak but specific signal was also observed in crude synaptosomes (P2) and other synaptic membrane fractions, e.g. P3 and LP2, while the synaptic plasma membrane fraction primarily appears devoid of Sumo2 conjugates (Figures S4B and S4C).

Cellular fractionation experiments are often burdened with cross contamination between fractions that can limit interpretations to assessing relative enrichment in each fraction. Well-controlled immunofluorescent studies can overcome this limitation and provide spatial evidence for protein localization. As such, we used homozygous HA-Sumo2 mice to further characterize extranuclear Sumo2 by performing anti-HA immunolabeling of the hippocampal CA3 area, a region enriched in synapses (Figure S3). Brain sections from WT and HA-Sumo2 mice were immunolabeled for HA and neuronal markers for dendrites and synaptic compartments, and anti-HA immunosignals were quantified in HA-Sumo2 mice as compared to WT (Figure 3). Using confocal microscopy, we confirmed the presence of Sumo2 in extranuclear compartments, specifically along dendrites and in synapses (Figure 3A). Quantification of HA-Sumo2 immunosignals in DAPI-positive area confirmed the strong enrichment of Sumo2 conjugates in neuronal nuclei, in agreement with the well-known role of SUMOs as nuclear regulators (Figures 3A and 3B).^{37,38} In addition, HA-Sumo2 immunosignal was also observed in MAP2-positive regions and, strikingly, in synapses positive for Synapsin1, Shank2, Gephyrin, and/or VGlut1 (Figures 3C and 3D). Higher magnification images confirmed the presence of Sumo2 along MAP2 dendrites and at synapses (Figures 4 and S5). Quantification of the 3D object-based co-localization between the anti-HA immunosignal with the various synaptic markers confirmed the presence of Sumo2 at synapses, with HA-Sumo2 signal being significantly co-localized with Shank2, Synapsin 1, VGlut1, and vGAT (Figures 4C and S5, which shows a typical 3D reconstruction generated in Imaris). Co-localization of Sumo2 with Gephyrin was not obvious, most likely due to difficulties in immunolabeling inhibitory synapses with the method optimized for HA visualization. Altogether, our biochemical fractionation and immunofluorescence support the presence of Sumo2 at synapses.

HA-Sumo mice reveal convergent and contrasting neuronal Sumo1 and Sumo2 substrates

The observation that there are regional and subcellular differences between Sumo1 and Sumo2 indicate divergent roles for the Sumo paralogs in the mouse brain. To uncover such molecular differences between Sumo paralogs, we performed HA-tag immunoprecipitation, under denaturing conditions to break apart standard protein-protein interactions, from whole-brain lysate of WT, His₆-HA-Sumo1, and His₆-HA-Sumo2 mice, followed by mass spectrometric analysis to identify candidate targets of Sumo1 and Sumo2 *in vivo* (Figures 5A and S6A; Table S2). To rank proteins identified from the mass spectrometry dataset, we summated the total peptides from 4 replicates per genotype and then filtered proteins with >2-fold enrichment over proteins identified in the WT condition. Next, to determine high-stringency candidates, proteins were further filtered based on peptide counts being identified in at least 2/4 replicates in the His₆-HA-Sumo conditions, and no more than 1/4 replicate in the respective WT condition. Using these criteria, we identified 131 proteins enriched in the His₆-HA-Sumo1 and 75 proteins enriched in the His₆-HA-Sumo2 immunoprecipitations (Figures 5B and S6B; Table S2). Gene ontology and Reactome terms

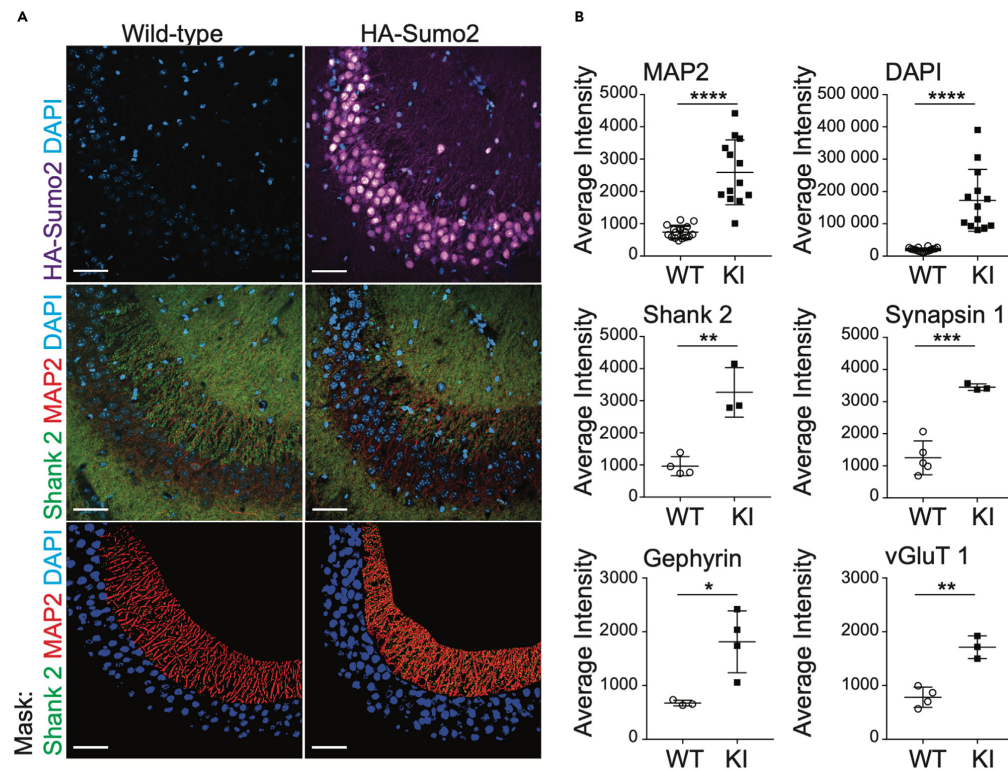


Figure 3. Extranuclear localization of Sumo2 in neurons of the hippocampal CA3 region

(A) Anti-HA (purple), DAPI (blue), MAP2 (red), and anti-Shank2 (green) immunostaining of wild-type (left) and HA-Sumo2 KI (right) hippocampal CA3 region. Scale bar: 50 μ m.

(B) Anti-HA signal in masked regions for each marker (exemplified for Shank2 in the bottom panel A) was quantified in both wild-type (WT) and HA-Sumo2 KI, and average intensity was calculated. N = 3, *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001. See also Figure S4.

related to protein SUMOylation process (Figure 5B) were significantly enriched in both datasets (Figures 5C and S6C; Table S2). Indeed, we identified critical components of the SUMOylation pathway including E1 SUMO ligases Sae1 and Uba2, the E2 SUMO ligase Ube2i, and E3 ligases Ranbp2,³⁹ Trim28,⁴⁰ and Pml,⁴¹ validating the use of our mouse model to identify molecular substrates involved with SUMOylation.

To determine whether we could identify specific interactors of Sumo2, we compared the relative fold change of peptides identified in the His₆-HA-Sumo2 immunoprecipitation to that of the His₆-HA-Sumo1 immunoprecipitation and found that there are shared, specific, and preferential interactors of Sumo2 vs. Sumo1 (Figures 5B, S6B, and S6D; Table S2). We selected several hits from our mass spectrometry screen for validation via Western blot (Figure 5D). Ranbp2 and Rangap1 are consistently identified among the most abundantly SUMOylated proteins^{7,42,43} and were indeed validated to be shared targets of Sumo1 and Sumo2 in the mouse brain. In addition, both proteins displayed a preference for Sumo1 modification in the mouse brain as previously described.⁴² Conversely, the E3 ligase and transcriptional repressor Trim28 displayed a preference for Sumo2 modification in the adult mouse brain. Interestingly, the transcription factor Prox1, involved in neurogenesis and a marker of granule cells in the dentate gyrus and cerebellum,⁴⁴ was one of the most abundantly SUMOylated proteins targeted specifically by Sumo2 in our screen. We further tested the sensitivity of the model to detect more modestly SUMOylated proteins

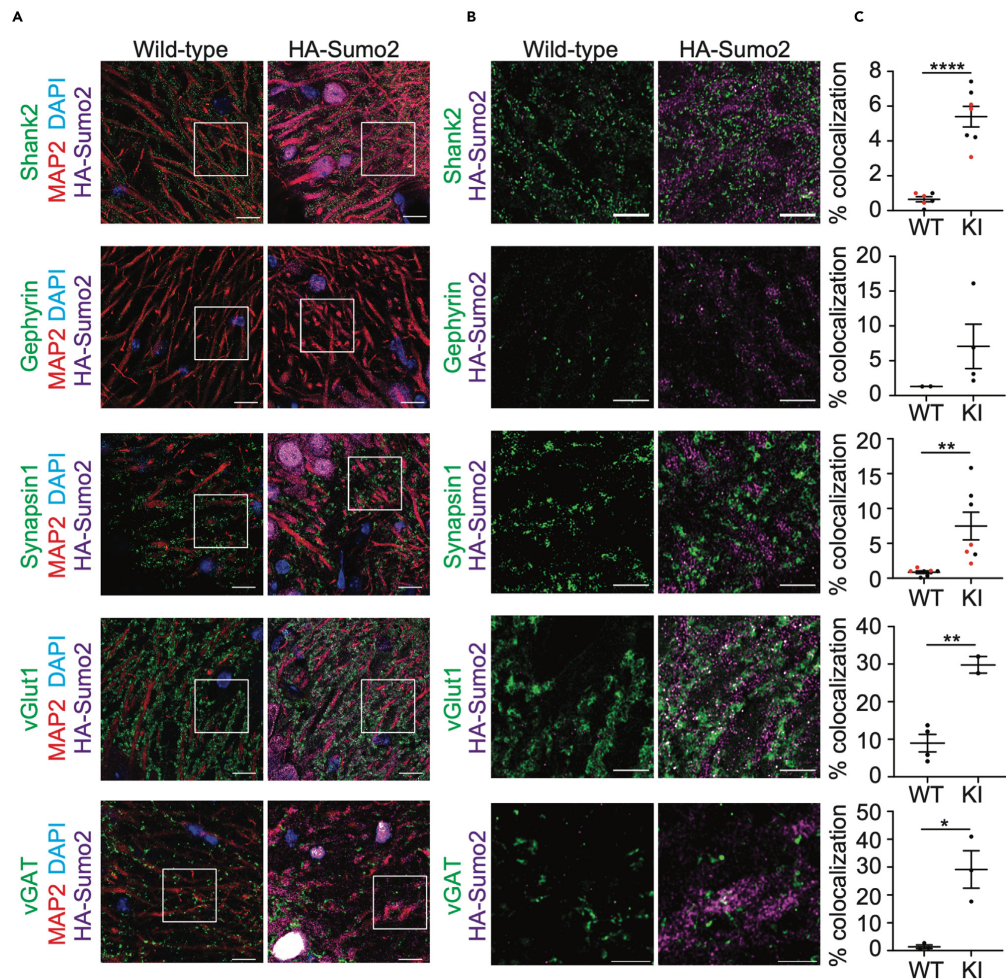


Figure 4. Sumo2 localizes at synapses in vivo

(A) High magnification images of wild-type (left) and HA-Sumo2 KI (right) hippocampal CA3 region labeled with anti-HA (purple), DAPI (blue), MAP2 (red), and synaptic markers (green) Shank2, Gephyrin, Synapsin1, vGlut1, and vGAT1. Scale bar, 10 μ m. (B) High-magnification images with equally scaled anti-HA intensity corresponding to the insets (white boxes) in A showing anti-HA (purple) immunostaining of wild-type (left) and HA-Sumo2 KI (right) hippocampal CA3 region immunolabelled with the synaptic markers (green) Shank2, Gephyrin, Synapsin1, vGlut1, and vGAT1. Scale bar: 5 μ m. N = 3. (C) The percentage of co-localization between each synaptic marker and the anti-HA signal was quantified in both wild-type (WT) and HA-Sumo2 KI. N = 3. Data are presented as mean $\pm$ SEM. *p < 0.05, **p < 0.005, ****p < 0.0001. See also Figure S5.

targeted by Sumo2 in the mouse brain, as measured by relative abundance in our immunoprecipitation coupled with mass spectrometry experiment. We confirmed that both histone deacetylase 2 and general transcription factor 2i (Gtf2i), also known as Tf2-i) were Sumo2 modified (Figure 5D). Because of the dynamic nature of SUMOylation and the fact that Sumo only targets a fraction of the total pool of most substrates, biologically meaningful interactions may be buried under strong, constitutively SUMOylated targets in our

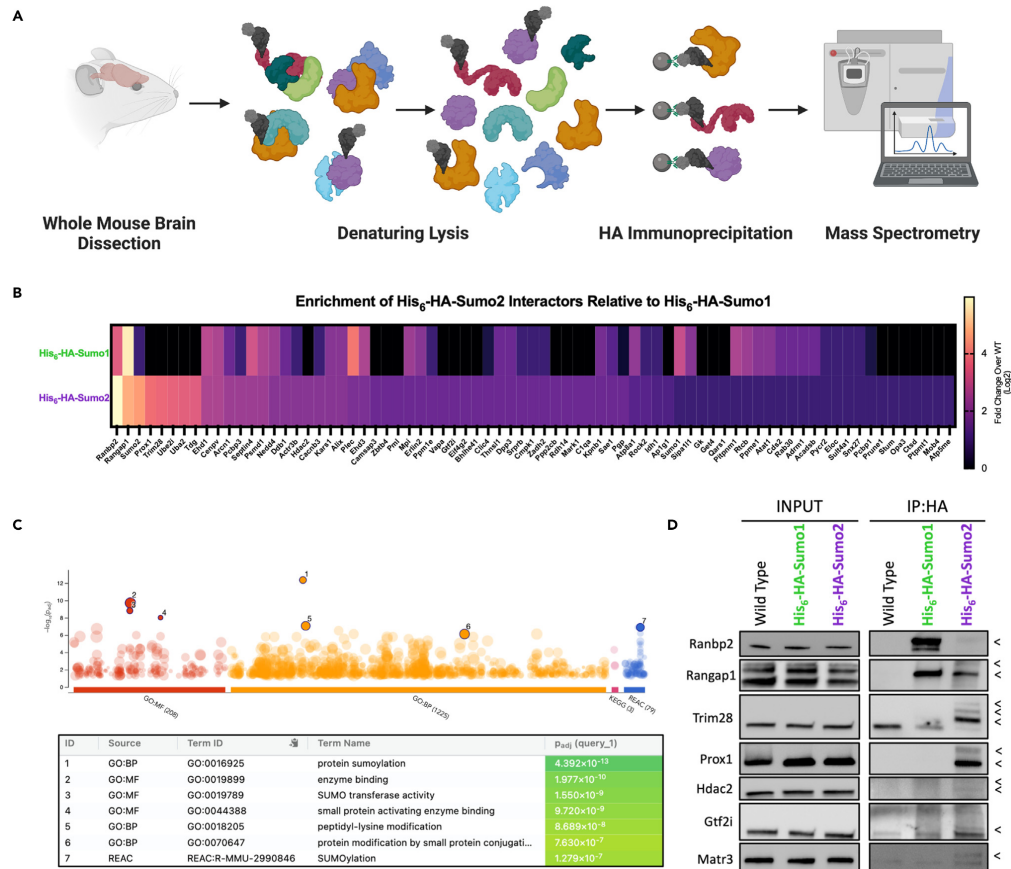


Figure 5. Neuronal Sumo2 has shared and distinct substrates compared to Sumo1 *in vivo*

(A) Schematic of approach to identify His₆-HA-Sumo interactors from the adult mouse brain via anti-HA affinity immunoprecipitation and mass spectrometry (N = 4). (B) Heatmap depicting the relative peptide abundance of His₆-HA-Sumo2 interactors relative to levels in His₆-HA-Sumo1 immunoprecipitation. (C) gProfiler2 Gene Ontology analysis for His₆-HA-Sumo2 interactors. (D) Anti-HA affinity immunoprecipitation followed by Western Blot analysis of His₆-HA-Sumo1 and His₆-HA-Sumo2 interactors as listed on the left side (N = 3–6). Black arrowheads indicate protein SUMOylation inferred by shift in molecular weight. See also Figure S6 and Table S2.

screen. As a proof of concept, we picked a hit with low enrichment in the His₆-HA-Sumo2 immunoprecipitation by expanding the filtering criteria to a >1.5-fold enrichment over WT: Matrx3 (Matrx3). We found that Matrx3 was selectively SUMOylated in the His₆-HA-Sumo2 pulldown (Figure 5D), indicating that even at milder cutoff thresholds, SUMOylated substrates identified by mass spectrometry can be validated in an orthogonal assay. Taken together, these results show that the His₆-HA-Sumo2 mouse model is effective to identify SUMOylated proteins, including proteins SUMOylated at relatively low levels *in vivo*, and can be used for targeted studies of protein substrates.

Subcellular localization of Sumo interactions in neurons

Sumo1 and Sumo2 predominantly reside in the nucleus, and unsurprisingly, many of the interactors identified here and in other studies of protein SUMOylation are nuclear proteins. However, previous studies

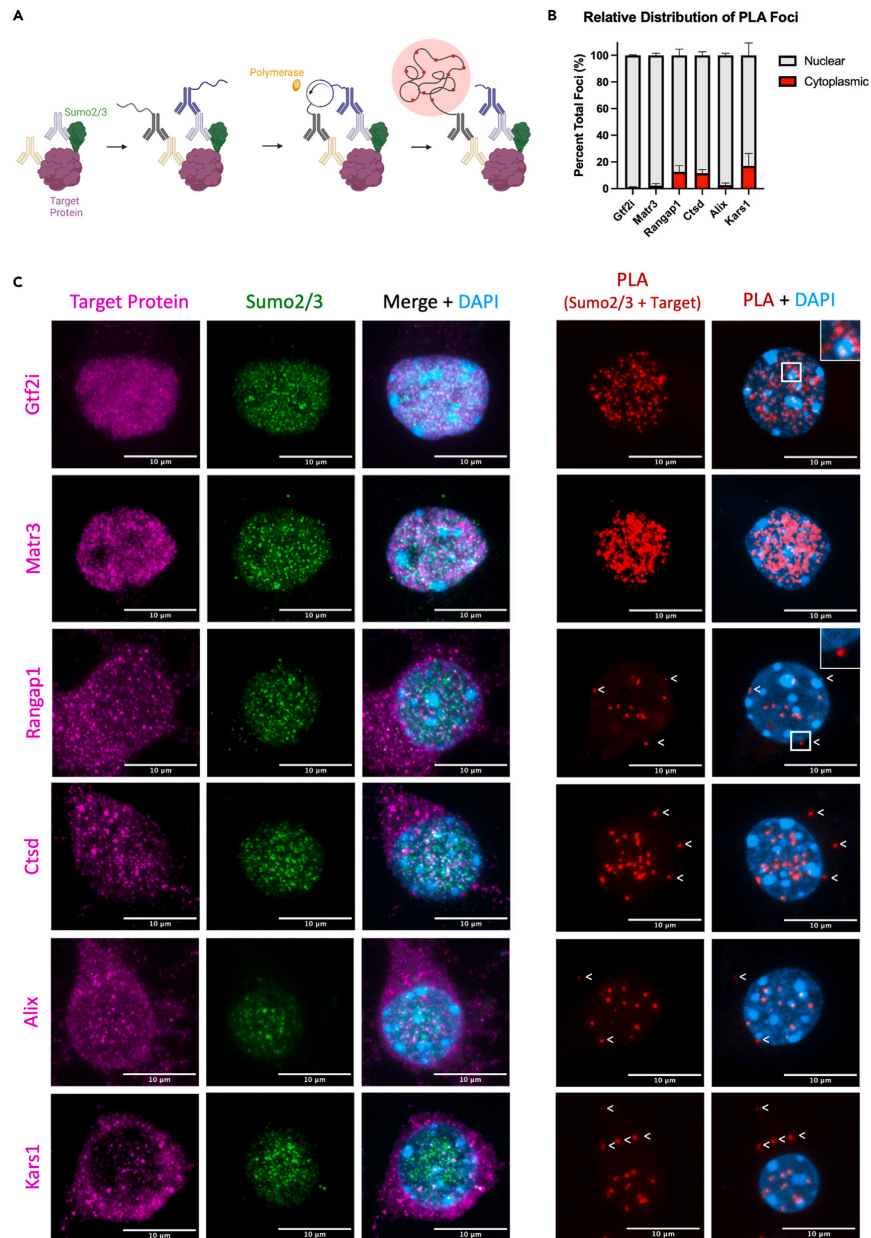


Figure 6. Established and newly identified Sumo2 substrates are present both in nuclear and extranuclear compartments in neurons

(A) Schematic of the proximity ligation assay (PLA) strategy for native Sumo2/3 and target proteins in WT primary cortical neurons. (B) The relative proportion of PLA foci between the selected target proteins and Sumo2/3 was quantified and normalized within the nucleus (gray) versus outside of the nucleus (red) (N = 3). (C) Representative Z-projected immunofluorescent images and PLA assays for selected target proteins identified from the mass spectrometry screen (N = 3). White arrowheads indicate cytoplasmic PLA foci. Scale bar: 10 μ m. Data are presented as mean $\pm$ SEM. See also Figure S7.

hinted at roles for SUMOylation outside of the nucleus.^{2,45–48} During the validation of whole-brain imaging, we observed that His₆-HA-Sumo2, while extensively localized throughout the nucleus, could also be observed within the somata and synapses of neurons *in vivo*, contrasting the predominantly nuclear membrane staining of His₆-HA-Sumo1 (Figures S3 and 3). The cytoplasmic localization of His₆-HA-Sumo2 in neurons indicates unique extranuclear roles *in vivo*. Indeed, we identified several predominantly extranuclear proteins via mass spectrometry in the anti-HA affinity purification from the His₆-HA-Sumo2 mice. To better visualize the subcellular localization of Sumo, we cultured primary cortical neurons from heterozygous His₆-HA-Sumo1, His₆-HA-Sumo2, and WT animals and co-stained them for HA and Map2 as a cytoplasmic marker. As previously observed, Sumo2 is predominantly distributed uniformly throughout the nucleus except for heterochromatic foci but is also observed at low levels in the cytoplasm (Figure S7A), contrasting with the anti-HA immunosignal observed for Sumo1, which predominantly localizes at the nuclear membrane. This indicates that Sumo2 specifically may play a larger role in regulating proteins outside of the nucleus.

To better assess the subcellular localization of neuronal SUMOylated proteins targeted by Sumo2, and to provide an additional level of validation in an orthogonal system, we used proximity ligation assays (PLA) against endogenous Sumo2/3 and targets of Sumo2 identified from our mass spectrometry screen to visualize interactions within WT primary cortical neurons (Figure 6A).^{49,50} In this assay, proteins colocalizing within 40 nm will produce a PLA signal suggesting a potential interaction. We selected proteins expected to interact with Sumo2/3 in the nucleus, Gtf2i, and Matr3, as well as expected cytoplasmic interactors Rangap1, Cttd, Alix, and Kars1 for additional validation (Figures 6B and 6C). Gtf2i is distributed almost exclusively throughout the nucleus and interactions between Sumo2/3 and Gtf2i, inferred by the presence of PLA foci, are seen throughout the nucleoplasm. Interestingly, PLA foci were often observed to cluster around constitutive heterochromatin (Figure 6C, inset), indicating a potential role for Sumo2 in regulating Gtf2i at the periphery of heterochromatin in neurons. Matr3 is also localized throughout the nucleus and to a lesser extent in the cytoplasm, consistent with its identification in cytoplasmic processing bodies.⁵¹ Interestingly, PLA signals for Matr3 and Sumo2/3 are robust, indicating extensive interactions between the two proteins. Immunofluorescence staining of Rangap1, Cttd, Alix, and Kars1 demonstrates these proteins predominantly reside outside of the nucleus (Figure 6C). PLA between Rangap1, Cttd, and Kars1 with Sumo2/3 display an increase in the proportion of cytoplasmic foci relative to nuclear foci (Figure 6B), in addition to a substantial decrease in total nuclear PLA foci compared to nuclear localized proteins Gtf2i and Matr3 (Figure S7B). Importantly, for all PLA assays conducted, negative controls did not elicit a PLA signal (Figure S7C). Taken together, our PLA analyses of WT neurons provide an additional layer of validation of Sumo2-substrate interactions. With the added benefit of providing spatial resolution to the interactions, PLA data support a role for neuronal Sumo2 outside the nucleus.

DISCUSSION

A constantly growing body of evidence demonstrates that SUMOylation plays key roles in the brain including neuronal development or learning and memory.^{18,20,45,52–54} Furthermore, SUMOylation is linked to various brain disorders, including stroke, autism spectrum disorder, epilepsy, schizophrenia, or neurodegenerative diseases, such as Alzheimer disease, Parkinson disease, and amyotrophic lateral sclerosis (ALS).^{55–62} However, despite a clear connection to neuronal function, SUMOylation in neurons has remained somewhat enigmatic, mainly due to technical challenges and limitations to study this PTM.³³ We generated His₆-HA-Sumo2 and HA-Sumo2 knockin mouse lines to overcome these technical limitations and to provide insight into the role of native SUMOylation *in vivo*. These mouse lines complement the previously generated His₆-HA-Sumo1 line,³² enabling the direct comparison of Sumo1 and Sumo2 biology *in vivo* and the elucidation of specific Sumo2 functions.

We observed that Sumo2 is more abundantly expressed throughout the CNS than Sumo1 and Sumo3 (Figure 1B). Interestingly, on both the RNA and protein levels, Sumo2 also displayed significant variations between CNS regions whereas Sumo1 levels did not significantly change. This may suggest that Sumo2

plays regional roles in the CNS. In addition, we observed that the pool of free Sumo2 is significantly greater than that of Sumo1 in all CNS regions, and levels of free Sumo2 significantly differ between regions (Figure 1C). As Sumo2 has been described to respond to stimuli, such as stressors, the pool of free Sumo2 may reflect the dynamic nature of SUMOylation in each region with cells requiring more Sumo2 to be readily available to respond dynamically in the cellular environment.⁶³ Future studies focusing on SUMO dynamics in neuronal populations may help to uncover paralog and cell-type-specific roles for SUMO-mediated regulation in the CNS.

Using whole-brain imaging upon HA epitope labeling in His₆-HA-Sumo1 and His₆-HA-Sumo2 mice, we found that Sumo1 and Sumo2 are broadly distributed throughout the mouse brain. More detailed anatomical analyses revealed distinct differences between Sumo1 and Sumo2 levels across brain structures and some divergence between Sumo1 and Sumo2 levels in the brain. Levels of His₆-HA-Sumo1 are generally similar between cortical layers, whereas His₆-HA-Sumo2 levels are higher in layers 2/3 (Figure S2D). In the hypothalamus, we found that Sumo levels vary across anatomical regions (Figure S2E). Interestingly, regions involved in circadian rhythm control, including the subparaventricular zone and suprachiasmatic nucleus, show high levels of Sumo2. These findings are consistent with previous reports indicating that Sumo1 and Sumo2 differentially affect circadian-clock-related proteins, such as PER2 and CLOCK, and support the notion of a role of SUMOylation in regulating circadian rhythms.^{36,64,65}

Importantly, we observed striking differences between the subcellular distribution patterns of Sumo1 and Sumo2. Western blot analysis of brain subregional fractions showed that Sumo2 is more abundant than Sumo1 (Figure 1C) and that Sumo2 conjugates, unlike Sumo1 conjugates, are prominently present in extranuclear fractions, including synaptic fractions (Figures S4B and S4C). However, brain subcellular fractionation is often unclear and nuclear material frequently leaks to non-nuclear fractions. Thus, immunofluorescence analysis is more powerful in determining the precise subcellular localization of Sumo-conjugated proteins. More specifically, anti-HA immunolabeling of brain material from HA-Sumo2 mouse brains and WT littermates as negative controls also demonstrated the presence of extranuclear-free Sumo2 and Sumo2 conjugates (Figures 3, 4, and S5). Quantitative analyses of anti-HA immunosignals confirmed a partial co-localization of Sumo2 with pre- and post-synaptic markers at excitatory and inhibitory synapses (Figures 3A, 3B, and 4A–4C). These data, which were stringently controlled using WT material as negative control, partly align with a recent report describing Sumo2 localization at synapses.³¹ On aggregate, our approach to study Sumo2 expression using the HA-Sumo2 mice allows for the specific assessment of Sumo2 immunosignals in neuronal sub-compartments and provides unequivocal evidence for the presence of Sumo2 at mammalian brain synapses. Thus, not only do Sumo1 and Sumo2 display differential expression and protein levels throughout the brain but are also localized and operational in different neuronal subcompartments. However, the functions of Sumo2 at synapses remain elusive. Importantly, there may be both direct (by Sumo-conjugation or -binding) and indirect roles for Sumo1 and Sumo2 in synaptic function that will have to be carefully assessed in the future to better understand how Sumo regulates synapse biology. In sum, the specificity provided by the His₆-HA-Sumo1 and (His₆-) HA-Sumo2 models enables direct comparisons between paralogs by overcoming key technical limitations. This will help to more accurately uncover specific roles for SUMOylation *in vivo*.

Sumo2 is the only functionally essential Sumo paralog, and, as mentioned, has key roles in neurodevelopment, nerve cell function, and neurological diseases.^{17,52,55,66–68} To uncover some of the unique clientele targeted by Sumo2 conjugation, we performed HA-tag immunoprecipitation paired with mass spectrometry. As SUMOylation results in the covalent interaction between Sumo and the target substrate, mouse brains were lysed under denaturing conditions to promote the dissociation of non-covalent protein complexes prior to immunoprecipitation to help identify bona fide SUMOylation substrates. Using stringent criteria, 75 proteins were identified to be conjugated to Sumo2, ~50% of which were selective to Sumo2. Conversely, using the same criteria, we identified 131 candidate Sumo1 substrates, 98 of which were selective to Sumo1. “Canonical” synaptic proteins were not among the hit lists in these experiments, which are likely related to the fact that experiments were performed in whole adult brains lysates, where levels of SUMOylated synaptic proteins may be too low due to dilution effects. The dynamics of SUMOylation may add additional complications in capturing synaptic SUMOylation events as physiological conditions may differentially affect when synaptic proteins become SUMOylated. Interestingly, we found that synaptic cytosol fractions contained increased levels of free Sumo2 which may further support the notion of highly dynamic SUMOylation at synapses, or non-covalent Sumo2 regulation in these



compartments. Ultimately, a dedicated proteomics approach focused on synaptic fractions may be necessary to specifically detect synaptic Sumo2 substrates and uncover specific roles for Sumo2 at synapses.^{56,57} Pathway analysis of the Sumo2 dataset revealed that gene ontology terms related to SUMOylation were significantly enriched in the dataset, validating this method to identify potentially SUMOylated substrates. Moreover, pathway analysis of the Sumo1 dataset revealed enrichment of proteins involved in protein transport consistent with roles for Sumo1 in nucleocytoplasmic shuttling.^{39,58,59} Prox1 was validated via Western blot as one of the strongest interactors specific to Sumo2 in the mouse brain. Interestingly, previous studies demonstrated that SUMOylation of Prox1 by Sumo1 occurs *in vitro* and in cancer cell lines.^{60,61} During development, Prox1 was found to be SUMOylated by Sumo1, and this interaction is critical for neurogenesis in the neural tube development. However, the role of Prox1 in the mature brain remains unclear, although some data indicate an involvement in adult neurogenesis.⁶² Due to the extent of the Prox1 and Sumo2 interaction observed in this study, further analysis may uncover key roles of SUMOylated Prox1 in the adult mouse brain. We further found that the RNA-binding protein Matr3 interacts with Sumo2 *in vivo*, indicating that even a mild enrichment over WT in our dataset may identify *bona fide* biological interactors. Interestingly, many of the computationally predicted SUMOylation sites in Matr3 (K588 and K843) reside within a structurally disordered region of the protein that also happens to be enriched for mutations causing amyotrophic lateral sclerosis.⁶⁹ Structurally disordered regions are thought to be critically involved in the formation of protein aggregates in neurodegenerative diseases and given the key role of SUMO in controlling protein solubility, and the recent links between SUMOylation and ALS, SUMOylation of Matr3 may provide key insights into Matr3 biology related to this disease.

SUMOylation occurs in a context-specific manner and can regulate protein localization within cells. One of the best characterized examples concerns the regulation of nucleocytoplasmic shuttling across nuclear pore complexes.^{39,58,59} However, SUMOylation can also regulate sub-compartmental localizations, such as protein targeting to the nucleolus or to heterochromatin.^{70–72} As Sumo2 was observed throughout the nucleus and outside the nucleus *in vivo* and in cultured neurons, we sought to determine the subcellular localization of Sumo2 interactions with hits identified via IP-MS using a PLA-based approach (Figure 6). Gtf2i was SUMOylated almost exclusively in the nucleus but interestingly appeared to cluster around heterochromatin. Gtf2i can function as both a transcriptional activator and repressor in response to various signals. For example, the specific splice isoforms β and Δ undergo changes in subcellular localization to differentially regulate the immediate-early gene *c-Fos* in response to growth factor signaling.⁷³ However, roles for Gtf2i in regulating heterochromatin remain elusive. SUMOylation may provide a dynamic mechanism in response to neuronal activity to regulate both targeted expression at the gene level and global transcriptional changes.⁷⁴ Finally, interactions between Matr3 and Sumo2/3 occurred broadly throughout the nucleus. However, interactions between Matr3 and Sumo2 in mouse brain occurred at relatively low levels based on immunoprecipitation assays. This discrepancy observed in the nucleus may be explained via non-covalent interactions with Sumo2 through larger protein complexes as Matr3 typically functions in complex with other DNA- and RNA-binding proteins.^{75–77} Extranuclear proteins Rangap1, Cttd, and Kars1, despite having a certain level of PLA signal within the nucleus, displayed an increase in the proportion of cytoplasmic PLA foci relative to nuclear PLA foci implying increased interactions with Sumo2/3 outside the nucleus. Indeed, Rangap1 plays roles in nuclear import, but can also be found to some extent within the nucleus.⁷⁸ PLA foci between Rangap1 and Sumo2/3 were often found near the nuclear membrane, likely at nuclear pore complexes (Figure 6C, inset). Cttd is a lysosomal protease predominantly localizing to lysosomes, thus interactions between Sumo2/3 and Cttd may suggest that its SUMOylation may play a role in lysosomal metabolism.⁷⁹ Finally, extranuclear PLA foci could be observed for the lysyl-tRNA synthetase Kars1, consistent with reports of Sumo interacting with tRNA-related proteins.^{80–82} Together, these results suggest that Sumo2 may play a variety of roles outside the nucleus in various subcellular compartments. While PLA assays provide spatial information of putative protein interactions (i.e. interactions occurring within 40 nm), this does not discount indirect interactions (e.g. protein complexes).^{49,50} Thus, differentiating between covalent SUMOylation, interactions with SUMO interacting motifs, and protein complexes containing SUMOs are not possible using this approach. Extensive localization and biochemical analyses, including arginine to lysine mutagenesis to block covalent SUMOylation, should be performed to properly assess how SUMO confers regulation on a specific target throughout the cell.

In sum, the novel (His₆-)HA-Sumo2 knockin mouse lines described here represent powerful tools to facilitate the study of protein SUMOylation via Sumo2 *in vivo*. The HA epitope tag allows for specific yet versatile

modes to detect and capture Sumo2 in its native context, without altering its function. Furthermore, these mouse models add to the previously generated His₆-HA-Sumo1 allele, and thus expand the Sumo knockin mouse toolkit to begin exploring differential SUMOylation in parallel systems. The versatility of these models extends to all other tissue systems, as Sumo proteins are broadly distributed beyond the brain.^{63,83} Beyond basic biology, these alleles can be used to explore disease processes, for example by crossing these mouse lines into disease models, thus enabling the study of the Sumo-linked disease proteome.⁶⁶ Ultimately, these tools will advance our understanding of the essential biological processes and potential disease targets regulated by SUMOylation.

Limitations of the study

While the (His₆)-HA-Sumo2 knockin mice help to overcome technical limitations in studying SUMO biology, including low antibody specificity, inability to differentiate between endogenous SUMO2 and SUMO3, and reliance on overexpression models, it cannot be completely excluded that the knockin of the (His₆)-HA-tag slightly affects Sumo2 function. Indeed, these mouse lines allow to characterize Sumo1 and Sumo2, but we also observed early lethality on C57BL/6J or C57BL/6N background, which was not seen on an FVB/N background. This indicates an interaction between the His₆-HA-Sumo2 knockin genotype and the genetic C57BL/6J or N set-up. Such strain effects are seen in many contexts, highlighting the importance of validating new genetic models using multiple systems and approaches. Furthermore, our observation that only Sumo2, but not Sumo1, is detectable at synapses, does not completely exclude a role for Sumo1 in regulating synapse function *in vivo*.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

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DECLARATION OF INTERESTS

The authors have no competing interests to declare.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-HA-Tag (C29F4) Rabbit mAb	Cell Signaling	Cat# 3724, RRID:AB_1549585
Anti-HA.11(16B12) Epitope Tag Mouse Ab	BioLegend	Cat# 901516, RRID:AB_2820200
Anti-SUMO2/3 (8A2) Recombinant Antibody	Abcam	Abcam Cat# ab81371, RRID:AB_1658424
Duolink in situ PLA probe anti-Rabbit PLUS	Sigma-Aldrich	Cat# DUO92002, RRID:AB_2810940
Duolink in situ PLA probe anti-Mouse MINUS	Sigma-Aldrich	Sigma-Aldrich Cat# DUO92004, RRID:AB_2713942
Chemicals, peptides, and recombinant proteins		
HA Synthetic Peptide	Sinobiological	Cat#PP100028-5
N-Ethylmaleimide	Sigma-Aldrich	Cat#E3876
Critical commercial assays		
Duolink in situ detection reagents red	Sigma-Aldrich	Cat#DUO92008
Experimental models: Organisms/strains		
His6-HA-Sumo1 Knock In Mouse	Marilyn Tirard ³²	N/A
His6-HA-Sumo2 Knock In Mouse	This paper	N/A
HA-Sumo2 Knock in Mouse	This paper	N/A
FVB/N Mouse	Charles River Laboratories	Strain Code 207
Oligonucleotides		
qPCR Mouse <i>Sumo1</i> FWD: CAGGAGGCAAAACCTTCAAC	Wang et al., 2014 ¹⁷	N/A
qPCR Mouse <i>Sumo1</i> REV: CTCCATTCCCAGTCTTTTCG	Wang et al., 2014 ¹⁷	N/A
qPCR Mouse <i>Sumo2</i> FWD: ACGAGAAACCAAGGAAGGA	Wang et al., 2014 ¹⁷	N/A
qPCR Mouse <i>Sumo2</i> REV: CTCCATTCCAACGTGTCAG	Wang et al., 2014 ¹⁷	N/A
qPCR Mouse <i>Sumo3</i> FWD: AGAAGCCCAAGGAGGGTGT	Wang et al., 2014 ¹⁷	N/A
qPCR Mouse <i>Sumo3</i> REV: CCTCGGGAGGCTGATCCT	Wang et al., 2014 ¹⁷	N/A
qPCR Mouse <i>Gapdh</i> FWD: GGAGAGTGTTCTCGTCCC	This study	N/A
qPCR Mouse <i>Gapdh</i> REV: ATGAAGGGGTCGTTGATGGC	This study	N/A
qPCR Mouse <i>Hprt1</i> FWD: TGATAGATCCATTCTATGACTGTAGA	This study	N/A
qPCR Mouse <i>Hprt1</i> REV: AAGACATTCTTCCAGTTAAAGTTGAG	This study	N/A
Software and algorithms		
Imaris Version 9.9.1	Oxford Instruments	https://imaris.oxinst.com/
Other		
Whole Brain Imaging/Analysis	Life Canvas Technologies	https://lifecanvastech.com

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Maxime Rousseaux (max.rousseaux@uottawa.ca).

Materials availability

HA-Sumo2 and His₆-HA-Sumo2 mouse lines will be provided upon reasonable request.

Data and code availability

- Mass spectrometry data is available from the lead contact upon request.
- Microscopy data reported in this paper will be shared by the [lead contact](#) upon request.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mouse lines

C57BL/6J.

FVB/N (WT)

His₆-HA-Sumo1 (FVB/N Background)

His₆-HA-Sumo2 (FVB/N Background)

HA-Sumo2 (FVB/N Background)

Sex and age of experimental mice

Experiments were performed using a mix of male and female mice to prevent sex biases. Cortical neuron cultures experiments included neurons dissected from individual embryos using a mixture of male and female cultures for all experimental conditions. At the high level of characterization, we do not expect sex effects to be observed however we acknowledge that future in depth analyses may uncover unique sex effects with regards to SUMOylation. All experimental animals were between 8 and 30 weeks of age. Specific age/sex information for whole brain imaging and mass spectrometry experiments can be found in Supplement [Tables S1](#) and [S2](#).

Mouse husbandry

His₆-HA-Sumo1 and His₆-HA-Sumo2 mice, kept on an FVB/N background, were housed with up to 5 mice per cage on a 12 h light–dark cycle. Mice were fed *ad libitum* and all husbandry was performed by the uOttawa Animal Care and Veterinary Services staff. All animal work for the His₆-HA-Sumo1 and His₆-HA-Sumo2 mouse lines were done under the breeding (CMMb-3009 and CMMb-3904) protocols approved under the uOttawa Animal Care Committee. Experiments performed on the HA-Sumo2 mice were done in accordance with the guidelines for the welfare of experimental animals issued by the State Government of Lower Saxony, Germany (LAVES). Animals were hosted in a pathogen free facility at the Max Planck Institute of experimental Medicine and were maintained in groups in accordance with European Union Directive 63/2010/EU and ETS 123 (individually ventilated cages, specific pathogen-free conditions, 21 ± 1°C, 55% relative humidity, 12 h/12 h light/dark cycle). Mice received food and tap water *ad libitum* and were provided with bedding and nesting material. Cages were changed once a week. Animal health was controlled daily by caretakers and by a veterinarian. Health monitoring (serological analyses; microbiological, parasitological, and pathological examinations) was done quarterly according to FELASA recommendations with either NMRI sentinel mice or animals from the colony. The mouse colony used for experiments did not show signs of pathogens. All experiments were performed during a light cycle.

–3', WT Allele: 160 bp product, KI Allele: 187 bp product). 20 μ L reactions were prepared using 1 μ L clean gDNA, primers (4 pmol final), and 5X Biozym Hot-Start Taq DNA Polymerase plus extra Mg^{2+} (Biozym 331620XL). Thermocycler parameters: 96°C for 3 min, (94°C for 30 s, 62°C for 60 s, 72°C for 60 s) repeated for 32 cycles, final denature at 72°C for 7 min. His₆-HA-Sumo1 mice were genotyped as previously described.³²

Biochemical analysis of adult mouse brain and spinal cord

Mice were anesthetized using isoflurane inhalation and sacrificed via decapitation. Brains were quickly isolated, dissected for regional protein analysis, and flash frozen on dry ice. Analysis of total Sumo1 and Sumo2/3 levels were performed on whole brain lysates. Spinal cords were removed using hydraulic extrusion.⁸⁵ Samples were thawed and immediately lysed using a Dounce homogenizer in RIPA buffer (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing 50 mM freshly prepared N-Ethylmaleimide (Sigma), 0.25% β -mercaptoethanol, and Xpert Protease (GenDEPOT) and Xpert Phosphatase (GenDEPOT) inhibitor cocktails. Whole brain lysates were lysed in 11 mL of RIPA buffer described above and centrifuged at 125,000 $\times$ g for 2 h at 4°C. Regional lysates were centrifuged at 21,000 $\times$ g for 20 min at 4°C and the supernatant was removed and suspended in 4X Laemmli buffer (BioRad) with 2-mercaptaethanol (BioRad) and boiled at 95°C for 5 min. Samples were run on 8% polyacrylamide gels and transferred onto 0.45 μ m nitrocellulose membranes at 340 mA for 90 min and stained with ponceau to normalize protein levels between lysates. Normalized lysates were run on a 4–15% GTx Mini-PROTEAN (BioRad) or Bolt™ 4–12% Bis-Tris Plus (Invitrogen) gel and transferred onto a 0.45 μ m nitrocellulose membrane at 340 mA for 2 h for western blot analysis. Densitometry was performed using BioRad Image Lab software and Fiji/ImageJ by normalizing the HA intensity to ponceau for High Molecular Weight (>70 kDa) and free Sumo levels.

Real time quantitative PCR (RT-qPCR) analysis

RNA was extracted from mouse brain homogenate using Trizol-Chloro-form extraction (Invitrogen User Guide: TRIzol Reagent version B.0). Briefly, mouse brains were homogenized in 3 mL of PEPI Buffer [5 mM EDTA, 1X protease inhibitor (GenDEPOT cat# P3100-020), in 1X PBS] using a Dounce homogenizer. 3% of homogenate was added to 1 mL of TRIzol Reagent (Fisher Scientific cat# 15-596-026) and RNA was isolated as per the user guide referenced above. For CNS regional RNA analysis, tissue samples were homogenized in 300 μ L of PEPI buffer using a pestle and passed through a 28 gauge insulin needle before RNA was extracted using RNeasy Kits (Qiagen cat# 74104). cDNA was synthesized using 5X All-in-One RT Master Mix (Bio Basic cat# HRT025-10) following manufacturer's instructions. RT-qPCR was performed using Green-2-Go qPCR Master Mix (Bio Basic cat# QPCR004-S) with 25 ng cDNA per reaction and primers targeting mouse Sumo paralogs¹⁷ Sumo1 (NM_009460.2) (Forward: 5'- CAGGAGGCCAAACCTTCAAC-3', Reverse: 5'- CTCCATTTCCAGTTCTTTCG-3'), Sumo2(NM_133354.2) (Forward: 5'- ACGAGAAACCCA AGGAAGGA-3', Reverse: 5'- CTCCATTTCCAACCTGTGCAG-3'), Sumo3(NM_001301672) (Forward: 5'- AGA AGCCCAAGGAGGGTGT-3', Reverse: 5'- CCTCGGGAGGCTGATCCT-3') and housekeeping controls *Gapdh* (NM_001289726) (Forward: 5'-GGAGAGTGTTCCTCTGTCCTCC-3', Reverse: 5'- ATGAAGGGGTCTG TTGATGGC-3') and *Hprt1* (NM_013556.2) (Forward: 5'-TGATAGATCCATTCCTATGACTGTAGA-3', Reverse: 5'-AAGACATTTCTTCCAGTTAAAGTTGAG-3'). Reactions were run on BioRad CFX96 thermocycler (protocol: 95°C for 5 min, 40 cycles of 95°C for 15 s and 60°C for 60 s, then melting curve). Sumo Ct values were standardized to the average of *Gapdh* and *Hprt1* Ct values.

Brain preparation for whole brain clearing and HA immunolabeling

Mice were sedated via intraperitoneal injection of 16.25 mg sodium pentobarbital (Euthanyl, DIN 00141704). Once sedated, mice were perfused using 10 mL 1X PBS + 10 U/mL heparin (Millipore Sigma, H3393-50KU) followed by 10 mL of freshly prepared 4% paraformaldehyde. Brains were carefully isolated and stored in 13 mL of 4% paraformaldehyde at 4°C overnight with gentle shaking. Brains were rinsed with 1X PBS then shipped to LifeCanvas Technologies (MA) in 1X PBS + 0.02% sodium azide.

Whole mouse brain processing, staining, and imaging

Whole mouse brains were processed using the SHIELD protocol (LifeCanvas Technologies⁸⁶). Samples were cleared for 1 day at 42°C then actively immunolabeled using SmartBatch+ (LifeCanvas Technologies) based on eFLASH technology integrating stochastic electrotransport⁸⁷ and SWITCH.⁸⁸ Each sample was labeled with 60 μ g anti-NeuN (Encor, MCA-1B7) and 36 μ g rabbit anti-HA-tag (Cell Signaling Technology



#3724) followed by fluorescently conjugated secondary antibodies in a 3:2 primary:secondary molar ratio (Jackson ImmunoResearch). Samples were incubated in EasyIndex (LifeCanvas Technologies) for refractive index of RI = 1.52 and imaged at 3.6X using a SmartSPIM axially-swept light sheet microscope (LifeCanvas Technologies). Images were tile corrected, de-striped, and registered to the Allen Brain Atlas (Allen Institute: <https://portal.brain-map.org/>). NeuN channels for each brain were registered to 8-20 atlas-aligned reference samples using successive rigid, affine, and b-spline warping (SimpleElastix: <https://simpleelastix.github.io/>). Average alignment to the atlas was generated across all intermediate reference sample alignments to serve as the final atlas alignment value per sample. Fluorescent measurements from the acquired images were projected onto the Allen Brain Atlas to quantify the total fluorescence intensity per region defined by the Allen Brain Atlas. These values were then divided by the volume of the corresponding regional volume to calculate the intensity per voxel measurements.

HA immunoprecipitation from mouse brain lysates

Immunoprecipitation was performed based on the previously established protocol.^{32,89} Briefly, mice aged 9-16 weeks old were anesthetized and sacrificed via decapitation and the brain was quickly removed and homogenized using a Dounce homogenizer in RIPA buffer (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing 50 mM freshly prepared N-Ethylmaleimide (Sigma), 0.25% β -mercaptoethanol, and Xpert Protease (GenDEPOT) and Xpert Phosphatase (GenDEPOT) inhibitor cocktails and boiled for 5 min at 95°C. Samples were lysed on ice for 20 min with vigorous vortexing every 5 min before ultracentrifugation at an average of 100,000 $\times$ g for 30 min at 4°C. Supernatants were removed and spiked with 50 mM N-ethylmaleimide before being added to 50 μ L of magnetic protein G Dynabeads (Invitrogen) pre-conjugated with 10 μ g mouse HA antibody (in house) per sample. Samples were then placed on rotator at 4°C for 1 h. Beads were washed three times in 10 mL of RIPA buffer containing 20 mM N-Ethylmaleimide, protease, and phosphatase inhibitors. Wash buffer was thoroughly removed, and beads were eluted using 75 μ g synthetic HA peptide (Sino Biological Inc.).

Sample preparation for mass spectrometry

Two-thirds of eluted protein sample from HA Immunoprecipitation were run on a 4-15% Mini-PROTEAN (Bio-Rad) gel to separate proteins and remove synthetic HA peptide. Samples were stained using Silver Stain for Mass Spectrometry Kit (Thermo Scientific, 24600). Lanes were cut into 6-7 gel slices per sample and stored in 1% acetic acid until analysis via LC-MS/MS.

Protein identification by LC-MS/MS

Proteomics analysis was performed at the Ottawa Hospital Research Institute Proteomics Core Facility (Ottawa, Canada). Proteins were digested in-gel using trypsin (Promega) according to the method of Shevchenko⁹⁰ but without the use of iodoacetamide for cysteine alkylation due to the treatment of 50 mM N-ethylmaleimide (Sigma) during the sample immunoprecipitation. Peptide extracts were concentrated by Vacufuge (Eppendorf). LC-MS/MS was performed using a Dionex Ultimate 3000 RLSC nano HPLC (Thermo Scientific) and Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific). MASCOT software version 2.7.0 (Matrix Science, UK) was used to infer peptide and protein identities from the mass spectra. The observed spectra were matched against *Mus musculus* sequences from SwissProt (version 2021-02) and against an in-house database of common contaminants. The results were exported to Scaffold (Proteome Software, USA) for further validation and viewing and will be uploaded to the ProteomeXchange Consortium.

Gene ontology analysis

Top His₆-HA-Sumo1 and His₆-HA-Sumo2 interactors were analyzed using gProfiler2 web tool (<https://biit.cs.ut.ee/gprofiler/gost>).⁹¹ Lists were arranged in descending order based on relative peptide abundance and analyzed as an ordered query for *Mus musculus* proteins using Benjamini-Hochberg FDR significance threshold with an alpha of 0.05.

Immunohistochemistry of mouse brain

Mice were anesthetized with 30 μ L of 120 mg/kg Euthanyl (DIN00141704) and then perfused with 10 mL 1x phosphate buffered saline (PBS) and 10 mL 4% paraformaldehyde (PFA). Brain and spinal cord tissue were collected and stored for 48 h in 4% PFA. Brain and spinal cord tissue were then dehydrated in 10%, 20% and

30% sucrose solutions for 48 h each before being flash frozen in -40°C isopentane for 1 min. Tissues were then sectioned at $20\ \mu\text{m}$ and -21°C on the Thermo Scientific HM 525 NX cryostat at the Louise Pelletier Histology Core at the University of Ottawa and stored free floating in $1\times$ PBS +0.02% NaN_3 at 4°C until use in staining. Brains tissue were incubated for 24 h in blocking buffer (1.5% Triton X-100, 5% cosmic calf serum in $1\times$ PBS), 24 h in primary antibody (1:500 HA-tag C29F4 rabbit monoclonal antibody (Cat: 3724S, Cell Signaling Technology), and 1 h in secondary antibody (1:500 Alexa Fluor 568 donkey anti mouse antibody, Cat: A10037, Lot: 1917938)) with DAPI (1:1,000 Millipore Sigma, D9542-1 MG). Tissue was washed 5 times for 5 min each in $1\times$ PBS between each treatment and mounted on Fisherbrand Superfrost Plus slides. Slides were left to dry for 24 h and covered with DAKO mounting medium (Cat: S3023, Lot: 11347938) and #1.5 coverslips.

For the analysis of the synaptic localization of Sumo2, HA-Sumo2 mouse brains were used (Figure S1A). Mice were anesthetized (250 mg/kg Avertin i.p.) and transcardially perfused with PBS and then with 4% (w/v) paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.4 at 4°C for 10 min. Brains were removed and post-fixed for 1 h at 4°C . The tissue was cryoprotected in 30% (w/v) sucrose in phosphate-buffered saline (PBS). Brains were frozen in the cryostat and sagittal $35\ \mu\text{m}$ sections were prepared with a cryostat and collected free-floating in PBS. For immunohistochemistry, sections were pre-incubated for 24 h in PB containing 3% horse serum (HS), 3% fish skin gelatin (FSG), and 0.3% Triton X-100, and were then incubated for 3 days at 4°C in primary antibodies diluted in PBS containing 3% HS, 3% FSG and 0.3% Triton X-100. After washing repeatedly in PBS and overnight, sections were incubated overnight in dye coupled secondary antibodies, repeatedly washed, and mounted on slides with Aquapolyount (Polysciences). The antibodies used are listed in Table S3.

Primary cortical neuron cultures

Pregnant mice were euthanized between at gestation E14.5–15.5 with 48 mg Pentobarbital Sodium (Bimeda-MTC, 8015E) delivered via intraperitoneal injection. Embryos were removed, washed in chilled PBS (Wisent Bioproducts, 311-010-CL), and cortices were isolated in chilled HBSS (Sigma Aldrich, H9394). Cortices were dissociated for 20 min with trypsin (Thermo Scientific, 90305) at 37°C before adding trypsin inhibitor and DNase solution to quench reaction. Cells were pelleted at $2,500\times g$ for 5 min at 4°C and washed with trypsin inhibitor with DNase solution. Cortical neurons were pelleted at $2,500\times g$ for 5 min at 4°C and resuspended in 1 mL Neurobasal outgrowth media (Thermo Scientific, 21103049), supplemented with B-27 (Thermo Scientific, 17504044), N-2 (Thermo Scientific, 21103049), $500\ \mu\text{M}$ L-Glutamine (Wisent Bioproducts, 609-065-EL), and 0.5% penicillin/streptomycin (GE Healthcare Life Sciences, SV30010) before plating. Cultures were maintained for 7 days *in vitro* with a half media change after 3–4 days.

Immunofluorescence in primary cortical neurons

Micro Coverglass #1.5 coverslips (Electron Microscopy Sciences) coverslips were pre-coated with poly-D-lysine ($50\ \mu\text{g}/\text{mL}$) overnight at 37°C , then washed with distilled water three times and air-dried at room temperature for at least 2 h. Primary mouse cortical neurons were seeded at 75,000 cells per coverslip and cultured as described in primary cortical neuron cultures. On day 7, neurons were fixed using 10% phosphate buffered formalin (Fisher Chemical, SF100-4) for 10 min followed by $3\times 5\text{-min}$ washes in 1 mL of $1\times$ PBS. Neurons were blocked in $500\ \mu\text{L}$ of blocking buffer (1.5% Triton X-100, 10% cosmic calf serum in $1\times$ PBS) for 1 h, then incubated in $300\ \mu\text{L}$ of primary antibody diluted in blocking buffer overnight at 4°C . The following day, the neurons were washed for $4\times 5\text{-min}$ washes in 1 mL of $1\times$ PBS then incubated in $300\ \mu\text{L}$ of secondary antibody diluted in blocking buffer for 2 h at room temperature. Next, the neurons were washed for $4\times 5\text{-min}$ washes in 1 mL of $1\times$ PBS, dried, and then placed on slides with Vectashield Antifade Mounting Medium with DAPI (MJS Biolynx Inc., H-1200). z stack images were obtained on a Zeiss AxioObserverZ1 LSM800 Confocal Microscope at $63\times$ magnification through a Z distance of $10\ \mu\text{m}$ per image using optimal $0.27\ \mu\text{m}$ spacing per slice. The dimensions were set to $1,024\times 1,024$ pixels.

Proximity ligation assay in primary cortical neurons

Primary mouse cortical neurons were cultured and fixed as described for immunofluorescence experiments. Individual coverslips for Proximity Ligation Assay experiments (PLA) were transferred to 12-well plates and outlined with a hydrophobic pen. Neurons were blocked using $40\ \mu\text{L}$ of Duolink blocking buffer (Sigma Aldrich, DUO82007) at 37°C for 1 h and were then washed for $3\times 5\text{-min}$ washes in 1 mL $1\times$ PBS. Next, the neurons were incubated in $300\ \mu\text{L}$ of primary antibody diluted in blocking buffer (1.5% Triton X-100, 10%



cosmic calf serum in 1X PBS) overnight at 4°C. The following day, the neurons were washed for 3 × 5 min washes in 1 mL of Duolink Wash Buffer A (0.01 M Tris-Base, 0.15 M NaCl, 0.05% Tween 20, pH 7.4) followed by incubation in 40 µL of Duolink PLA MINUS (Sigma Aldrich, DUO82004) and PLUS probes (Sigma Aldrich, DUO82002) at 37°C for 1 h. Duolink PLA probes were diluted in antibody diluent (Sigma Aldrich, DUO82008) at a 1:5 dilution. Neurons were washed for 3 × 5 min washes in Duolink Wash Buffer A and then incubated in 40 µL of ligase (Sigma Aldrich, DUO82027) at 37°C for 30 min. Ligase was diluted in 1X ligation buffer (Sigma Aldrich, DUO82009) at a 1:40 dilution. Neurons were washed for 3 × 5-min washes in Duolink Wash Buffer A and then incubated in 40 µL of polymerase (Sigma Aldrich, DUO82028) at 37°C for 90 min. Polymerase was diluted in 1X amplification buffer (Sigma Aldrich, DUO82011) at a 1:80 dilution. Then, neurons were washed 2 × 10 min in Duolink Wash Buffer B (0.2 M Tris-Base, 0.1 M NaCl, pH 7.5) and then again in 1 mL of Duolink Wash Buffer B diluted at 1:100 for 1 min. Coverslips were briefly air dried and then mounted on slides using Vectashield Antifade Mounting Medium with DAPI. z stack images were obtained on a Zeiss AxioObserverZ1 LSM800 Confocal Microscope at 63× magnification with a 5× digital zoom through a Z distance of 10 µm per image using optimal 0.27 µm spacing per slice with dimensions set to 512 × 512 pixels. Images were analyzed and quantified using the Spots function on the Imaris (ver. 9.9.1 Bitplane, Switzerland) software. Localization of the foci (nuclear versus cytoplasmic) was determined using the Orthogonal Views function for co-localization with DAPI signal.

Tissue lysis

Dissected mouse organs were lysed in 1X RIPA buffer (9.1 mM Na₂HPO₄, 1.7 mM NaH₂PO₄, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1X protease inhibitor (GenDEPOT, P3100), 1X phosphatase inhibitor (GenDEPOT, P3200) and 50 mM N-ethylmaleimide (Sigma-Aldrich, E3876-5G) using a Dounce homogenizer. Tissue lysates were centrifuged at ~21,000 × g for 15 min at 4°C and the resulting supernatant was transferred into a fresh microcentrifuge tube.

Subcellular fractionation

Western blot analysis of brain subcellular fraction was performed as described previously.^{32,92}

Western blot analysis

SDS-PAGE was performed with standard discontinuous gels or with commercially available 4%–12% Bis-Tris gradient gels (Invitrogen). Western blots were probed using primary and secondary antibodies as indicated in Table S3. Blots were routinely developed using enhanced chemiluminescence (GE Healthcare) and imaged using an INTAS ECL Chemostar PLUS Imager HR 6.0. For quantitative Western blotting, transferred proteins were visualized using the total protein stain MemCode (Invitrogen). Western blot signals were visualized using an INTAS ECL Chemostar PLUS Imager HR 6.0 apparatus and quantified using ImageJ. For normalization, a ratio of the antibody signal relative to the total protein stain as revealed by the MemCode was performed.

Confocal imaging and image analysis

MAP2, HA-Sumo2, DAPI and the respective synaptic marker fluorescence intensities were acquired from the hippocampal CA3 pyramidal layer and the *stratum radiatum*. Multi-channel z-stacks of 338 × 338 µm-sized fields of view were acquired with a 40× oil immersion objective (1.3 NA) of a Nikon Eclipse Ti confocal microscope equipped with a Yokogawa W1 spinning disk and an Andor iXon Ultra 888 EMCCD camera. A custom macro written within the FIJI software package (Schindelin et al., 2012) automatically identified the z-plane of highest HA-Sumo2 intensity, out of which all channels were extracted. The DAPI, MAP2 and synaptic marker channel of this z-plane were segmented with the Trainable WEKA Segmentation FIJI plugin (Arganda-Carreras et al., 2017), resulting in binary masks (Figure 3A, bottom row) that were subsequently used to extract the respective HA-Sumo2 mean intensity.

To more closely investigate HA-Sumo2 co-localization with the respective synaptic markers, high-resolution, multi-channel fields of view (x, y: 30, 30 µm) of the respective stains within the Mossy fiber region were acquired using 40× oil-immersed objective (NA = 1.4) of a Zeiss LSM 880 confocal laser scanning microscope. Oversampled (x, y, z: 30, 30, 100 nm), 1.2 µm-sized z-stacks within the mossy fiber region were acquired for each fluorescent channel. Signal to noise ratio and resolution were subsequently enhanced and sample geometry was corrected with the SVI Huygens deconvolution software package (ver. 22.04, Hilversum, Netherlands). To obtain object-based co-localization information, surfaces depicting



either HA-Sumo2 or the respective marker were generated with an Imaris (ver. 9.9.1 Bitplane, Switzerland) batch workflow, which automatically and identically processed the three-dimensional fields-of-view originating from WT or knock-in animals. All HA-Sumo2 and the respective synaptic marker surface objects were created, yielding the total object number for the respective stain (Figure S5- top and middle rows of A and B). Synaptic marker objects were designated as collocating with the HA-Sumo2 objects only if they overlapped with HA-Sumo2 objects and if they contained a minimum HA-Sumo2 average intensity (Figure S5, bottom rows of A and B). Data resulting from both analysis workflows were assembled and quantified with the KNIME software package (ver. 4.5.2, Zurich, Switzerland).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical tests were performed using PRISM 9.4. Test type was picked based on the number of comparisons made. Levels of statistical significance are indicated in figure legends.

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Contribution: I helped with mouse handling, tissue acquisition, and analysis. My overall contribution to this paper was about 15%.

ARTICLE OPEN



Genetic and pharmacological reduction of CDK14 mitigates synucleinopathy

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Parkinson's disease (PD) is a debilitating neurodegenerative disease characterized by the loss of midbrain dopaminergic neurons (DaNs) and the abnormal accumulation of α -Synuclein (α -Syn) protein. Currently, no treatment can slow nor halt the progression of PD. Multiplications and mutations of the α -Syn gene (*SNCA*) cause PD-associated syndromes and animal models that overexpress α -Syn replicate several features of PD. Decreasing total α -Syn levels, therefore, is an attractive approach to slow down neurodegeneration in patients with synucleinopathy. We previously performed a genetic screen for modifiers of α -Syn levels and identified CDK14, a kinase of largely unknown function as a regulator of α -Syn. To test the potential therapeutic effects of CDK14 reduction in PD, we ablated Cdk14 in the α -Syn preformed fibrils (PFF)-induced PD mouse model. We found that loss of Cdk14 mitigates the grip strength deficit of PFF-treated mice and ameliorates PFF-induced cortical α -Syn pathology, indicated by reduced numbers of pS129 α -Syn-containing cells. In primary neurons, we found that Cdk14 depletion protects against the propagation of toxic α -Syn species. We further validated these findings on pS129 α -Syn levels in PD patient neurons. Finally, we leveraged the recent discovery of a covalent inhibitor of CDK14 to determine whether this target is pharmacologically tractable in vitro and in vivo. We found that CDK14 inhibition decreases total and pathologically aggregated α -Syn in human neurons, in PFF-challenged rat neurons and in the brains of α -Syn-humanized mice. In summary, we suggest that CDK14 represents a novel therapeutic target for PD-associated synucleinopathy.

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INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disease that affects over 10 million individuals worldwide [1, 2]. Individuals with PD present with motor symptoms such as bradykinesia, rigidity, shuffling gait, and resting tremor, as well as non-motor symptoms such as constipation, anosmia, and sleep disturbances [3–5]. Neuropathologically, PD is characterized by the loss of dopaminergic neurons (DaNs) in the *substantia nigra pars compacta* (SN), as well as the accumulation of α -synuclein (α -Syn) containing inclusions, termed Lewy bodies and Lewy neurites (collectively: Lewy pathology) in surviving neurons [6–8]. Current treatments address motor deficits, but are less effective on non-motor aspects of the disease and cannot slow down nor halt neurodegeneration in PD [9]. Therefore, identifying new 'druggable' targets for PD is clearly warranted. In addition to being abundantly present in Lewy pathology, point mutations and multiplications in the gene encoding α -Syn, *SNCA*, underlie monogenic variants of PD [10, 11]. Increased levels of *SNCA*

mRNA are also observed in laser-captured SN DaNs from PD patients [12], and animal models that overexpress α -Syn replicate several features of PD [13–15]. Thus, there is a clear link between increased α -Syn dosage and PD pathogenesis, highlighting the crucial role of α -Syn in the manifestation of PD [16–19].

Since α -Syn dosage is linked to PD, decreasing total α -Syn levels may be a feasible approach to mitigate neurodegeneration in PD patients, regardless of whether oligomeric or fibrillar α -Syn is the toxic culprit. *Snca*-knockout (KO) mice are viable and fertile but display mild cognitive impairments, suggesting that a modest amount of cerebral α -Syn is required to accomplish its physiological role in the synapse [20, 21]. Titration of excessive α -Syn levels in a non-invasive manner would thus be beneficial in treating a chronic neurodegenerative disease like PD. Specifically, an orally available drug capable of mitigating α -Syn toxicity could offer a minimally invasive approach, a feature particularly important in treating a chronic illness. A pooled RNA interference screen investigating 'druggable' modifiers of α -Syn levels identified

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cyclin-dependent kinase 14 (CDK14, a.k.a. PFTK1; Cdk14 or Pftk1 in mice) as a regulator of α -Syn [22]. CDK14 is a brain-expressed protein kinase with a largely unknown biological function [23, 24]. Its expression is upregulated in certain cancers, such as esophageal and colorectal cancer, for which it has generated attention as a therapeutic target [25, 26]. In fact, this has led to the recent development of FMF-04-159-2, a potent, covalent inhibitor of CDK14 [27].

Since decreasing CDK14 leads to a mild reduction in endogenous α -Syn levels [22], we hypothesize that genetic and pharmacological inhibition of CDK14 reduces α -Syn pathology and PD-like phenotypes in mice and human cells. To test this hypothesis, we examined the consequence of *Cdk14* reduction on PD-like features in the preformed α -Syn fibrils (PFFs)-induced PD mouse model. We further explored the role of CDK14 in mediating α -Syn spread in primary neurons. We also tested the effect of CRISPR/Cas9-mediated reduction of *CDK14* in human neurons carrying the PD-linked *SNCA* A53T mutation. Lastly, using the covalent CDK14 inhibitor, we investigated if pharmacological inhibition of CDK14 is sufficient to decrease α -Syn levels in rodent and human neurons. In summary, we show that decreasing CDK14, both genetically and pharmacologically, reduces α -Syn accumulation, spread, and modifies α -Syn aggregation.

MATERIALS AND METHODS

Mouse strains

Cdk14^{+/−} mice were generated by the Gene Targeting and Transgenic Facility of Texas A&M Institute for Genomic Medicine (TIGM), on a mixed (129/SvEvBrd (RRID:MGI:5654530) × C57BL/6 (RRID:MGI:2159769)) background (as described previously [28]. *Cdk14*^{+/−} mice were backcrossed 14 times to the C57BL/6NCrl (RRID:MGI:2683688) background prior to experimentation. *Cdk14*^{+/−} mice were paired to generate *Cdk14*^{−/−} mice (RRID:MGI:7547243). *Snca*^{−/−} founder mice were provided by Matthew Farrer (University of Florida, Gainesville, USA) and PAC α -Syn^{A53T} TG founder mice (db1-PAC-Tg(*SNCA*^{A53T})/^{+/+}, *Snca*^{−/−}, [29]) were provided by Robert L. Nussbaum (University of California, San Francisco, USA). Experiments were conducted with female and male mice and mice were genotyped using genomic DNA extracted from ear or tail tissue (genotyping protocol available upon request).

Stereotactic PFF injections

Endotoxin-free recombinant α -Syn fibril preparations were stored at −80°C before usage [30, 31]. On the day of usage, fibrils were thawed and sonicated in a sonicator water bath to generate PFFs (Covaris S220, 18 W peak incident power, 20% duty factor, 50 cycles per burst, 150 s). For transmission electron microscopy (TEM) analysis 0.2 mg/mL PFF samples were stained with Uranyl Acetate and imaged on an FEI Tecnai G2 Spirit Twin TEM (Centre for Advanced Materials Research [CAMA], University of Ottawa). Six-month-old mice were deeply anesthetized with isoflurane, and 1 μ L mouse PFFs (5 mg/mL) or sterile saline (0.9% NaCl) was unilaterally delivered into the dorsal striatum of the right hemisphere at these coordinates relative to bregma: −2 mm medial-lateral; +0.2 mm antero-posterior and −2.6 mm dorso-ventral. Injections were performed using a 2 μ L syringe (Hamilton Company, Reno, NV, USA) at a rate of 0.1 μ L/min (min) with the needle left in place for at least 3 min before its slow withdrawal. After surgery, animals were monitored, and post-surgical care was provided. Behavioral experiments were performed 6 months post-injection, followed by the collection of the brains at 7 months post-injection. <https://doi.org/10.17504/protocols.io.dmgp3pdjvzp/v1>.

Intracerebroventricular administration of the CDK14 inhibitor FMF-04-159-2

Alzet® Mini-Osmotic Pumps (model 2004) were loaded with FMF-04-159-216 (R&D Systems 7158, Minneapolis, MN, USA) 1.47 μ g/ μ L in vehicle solution containing 8% DMSO (Fisher Scientific, BP231, Hampton, NH, USA), 2% Tween 80 (Fisher Scientific, BP338-500) and 90% ddH₂O) 16 h before stereotactic surgeries. Brain infusion catheters with 3.5 cm long catheter tubing (Alzet® Brain Infusion Kit) were attached as per manufacturer's instructions. Brain infusion assemblies were incubated at 37°C in sterile saline until implantation. 4-month-old PAC α -Syn^{A53T} TG

were deeply anesthetized with isoflurane for the stereotactic implantation of brain infusion assemblies. FMF-04-159-2 (release rate of 0.35 mg/kg/day) or its vehicle solution was continuously administered into the cerebral ventricles (coordinates relative to bregma: −1.1 mm medial-lateral; −0.5 mm antero-posterior and −3 mm dorso-ventral) for 28 days with the brain infusion catheter attached to the skull and the connected pump in a subcutaneous pocket of the mouse's back. The body weight of mice was measured and their activity, neurological signs, facial grimace, coat condition, and respiration were scored (from 0 to 3) within 28 days after the surgery. Mice were sacrificed and organs were collected on the 28th day of the administration period. <https://doi.org/10.17504/protocols.io.x54v9pqy1g3e/v1>.

Behavioral experiments

Grip strength tests were performed by holding the mice at an automatic grip strength meter (Chatillon DFE II, Columbus Instruments, Columbus, OH, USA) allowing them to grip the grid of the device with their fore- and hindlimbs. Then, mice were gently pulled back by their tail until they released the grip. Force exerted by the mouse during its removal from the grid, as measured by gram force (g), was evaluated 5 times per mouse. For nesting behavior tests, mice were singly caged overnight (16 h) with a 5 cm × 5 cm cotton nestlet in a clean cage. Produced nests were scored on a scale from 1 to 5 as previously described [32]. For the tail suspension test, the tails of the mice were taped to a metal bar attached to the ENV-50STS Load Cell Amplifier and DIG-735 cabinet with high pass filter set to 1 Hz (Med Associates, Fairfax, VT, USA). The time of immobility was tested over 6 min. For the elevated plus maze test, mice were placed in the center of a maze consisting of two arms (6 cm × 75 cm), one open and the other enclosed. Over 10 min, the number of open arm entries was tracked with Ethovision software (Noldus Information Technology, Leesburg, VA, USA) and normalized to the total amount of arm entries. For the Y maze test, mice were placed in the center of the Y maze, where the three arms meet and given 8 min to explore. The number of arm alternations is measured relative to total arm entries using Ethovision software. For the open field test, mice were placed in a 45 cm × 45 cm open top cage and locomotion was automatically tracked with an overhead-mounted camera connected to a computer equipped with Ethovision tracking software (Noldus Information Technology). Open field motor activity was recorded over 10 min. <https://doi.org/10.17504/protocols.io.b5qzq5x6>. For the hanging wire test, mice were placed on a wire cage lid which was gently turned upside down over a cage, followed by the recording of the latency to falling from the lid. Mice were given three consecutive training trials, followed by three test trials. The average latency to fall was normalized to the body weight of the mouse. Pole tests were performed by placing the mice on the top of a vertical pole (8 mm diameter and 55 cm height) with a rough surface. Mice were placed vertically, on the top of the pole, and the time required for turning was recorded. The mean time to turn was calculated from 5 consecutive trials for each mouse. <https://doi.org/10.17504/protocols.io.b5sqg6dw>. For the rotarod test, mice were placed on a rotating, textured rod (ITC Life Science, Woodland Hills, CA, USA), with the speed gradually increasing from 4 to 40 rpm over 5 min. The latency to fall from the rotating rod was recorded for every mouse. Four trials per day with 10 min inter-trial intervals were performed for three consecutive days. <https://doi.org/10.17504/protocols.io.b5srq6d6>.

Tissue harvesting and processing

For biochemical approaches mice were anesthetized with isoflurane (Fresenius Kabi, CP0406V2, Bad Homburg, Germany), and decapitated. Brain tissue of 5-month-old PAC α -Syn^{A53T} TG mice was weighed and lysed 1:3 (w/v) in PEPI buffer (5 mM EDTA, protease inhibitor [GenDEPOT, P3100, Katy, TX, USA] and phosphatase inhibitor [GenDEPOT, P3200] in PBS) with a Dounce homogenizer. Samples were further lysed 1:6 (w/v) using the tissue weight in TSS Buffer (140 mM NaCl, 5 mM Tris-HCl), then TXS Buffer (140 mM NaCl, 5 mM Tris-HCl, 0.5% Triton X-100), and SDS Buffer (140 mM NaCl, 5 mM Tris-HCl, 1% SDS), as previously described [33].

For immunohistology with paraffin sections, mice were anesthetized with 120 mg/kg Euthanyl (DIN00141704) and intracardially perfused with 10 mL of PBS, followed by 20 mL of 10% Buffered Formalin Phosphate (Fisher Scientific, SF100-4). Brains were isolated and fixed in 10% Buffered Formalin Phosphate at 4°C for at least 24 h. After dehydration by 70%, 80%, 90%, and 100% ethanol and clearing by Xylenes, brains were infiltrated and embedded in paraffin (Louise Pelletier Histology Core Facility, University of Ottawa). Brains were sectioned at 5 μ m.

SDS-PAGE and mouse protein immunoblots

4X Laemmli buffer (Bio-Rad, 1610747, Hercules, CA, USA) with 20% 2-mercaptoethanol (Bio-Rad, 1610710) was added to cleared protein and boiled at 95 °C for 5 min. Protein samples were loaded on a 12% SDS-PAGE gel in the Mini-PROTEAN Tetra Cell (Bio-Rad, 165-8000). Protein was then transferred to a 0.2 µm nitrocellulose membrane (Bio-Rad, 1620112) using the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, 1703930) in Tris-Glycine buffer with 10% methanol (Fisher Scientific, A412P) at 340 mA for 90 min at 4 °C. Membranes were then blocked in 5% milk in 1X TBS-T for 1 h at room temperature followed by overnight incubation in primary antibody against pSer129 (pS) α-Syn (1:2000, Abcam ab51253, RRID:AB_869973), α-Syn (1:2000, BD Biosciences 610787, RRID:AB_398108), human α-Syn (1:1000, Abcam ab138501, RRID:AB_2537217), CDK14 (1:500, Santa Cruz Biotechnology sc-50475, RRID:AB_2163226), Ubiquitin (1:1000, Cell Signaling Technology 3936, RRID:AB_331292), LC3B (1:2000, Novus NB100-2220, RRID:AB_10003146), pSer1490 LRP6 (1:2000, Cell Signaling Technology 2568, RRID:AB_2139327), LRP6 (1:2000, Cell Signaling Technology 2560, RRID:AB_2139329) and GAPDH (1:40,000, Protein-tech, 60004-1-Ig, RRID:AB_2107436) diluted in 2% BSA, 0.02% Na₃ in 1X TBS-T. Next, membranes were washed in TBS-T, followed by incubation in secondary antibody (peroxidase-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Labs 711-035-152, RRID:AB_10015282) or donkey anti-mouse IgG (Jackson ImmunoResearch Labs 715-035-150, RRID:AB_2340770), both at 1:10,000 diluted in 5% milk in TBS-T) for 1 h at RT. Membranes were washed again in TBS-T, bathed in enhanced chemiluminescent reagent (Bio-Rad, 1705061), imaged using the ImageQuant LAS 4100 Imaging system (GE) and quantified using Bio-Rad Image Lab 6.1 software (<http://www.bio-rad.com/en-us/sku/1709690-image-lab-software>, version 6.1, RRID:SCR_014210). <https://doi.org/10.17504/protocols.io.b5wkq7cw>.

Dopamine measurements with liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS)

Striatal tissue punches from 3 mm thick brain sections from 12-month-old PFF-treated mice were weighed and submitted to The Metabolomics Innovation Centre (TMIC, Edmonton, AB, Canada) for analysis. Samples were homogenized in 50 µL of tissue extraction buffer, followed by centrifugation. Dopamine content in µM was analyzed by reverse-phase LC-MS/MS custom assay in combination with an ABSciex 4000 QTrap® tandem mass spectrometer (Applied Biosystems/MDS Analytical Technologies, Foster City, CA, USA) using isotope-labeled internal standards. The assay utilizes a 96 deep well plate with a filter plate attached on top. Samples were thawed on ice, vortexed and centrifuged at 13,000 × g. 10 µL of sample was loaded in the center of the filter and dried in a stream of nitrogen. Following derivatization by phenyl isothiocyanate and drying of filter spots, dopamine content was extracted by adding 300 µL of extraction solvent, centrifugation into the lower collection plate and dilution by MS running solvent. Mass spectrometric analysis was performed with the ABSciex 4000 QTrap® tandem mass spectrometer in combination with an Agilent 1260 series UHPLC system (Agilent Technologies, Palo Alto, CA, USA). Samples were delivered by an LC method followed by a direct injection method. Data was analyzed using Analyst 1.6.2 (<https://sciex.com/products/software/analyst-tf-software>, version 1.6.2, RRID:SCR_015785) and expressed as dopamine concentration relative to tissue weight.

Histology

For Diaminobenzidine (DAB) antibody staining, paraffin sections were deparaffinized in xylenes and rehydrated in a series of decreasing ethanol (100%, 90%, 70%, 50%) followed by antigen retrieval in sodium citrate buffer (2.94 g sodium citrate, 0.5 mL Tween 20 in 1 L PBS, pH6) at 80 °C for 2 h and quenching of endogenous peroxidase with 0.9% H₂O₂ in PBS for 10 min. Sections were blocked in blocking buffer (0.1% Triton X-100, 10% normal horse serum in PBS) and incubated in primary antibody (pSer129 α-Syn, 1:500, Abcam ab51253, RRID:AB_869973 or tyrosine hydroxylase, 1:500, Millipore B152, RRID:AB_390204) overnight at 4 °C. Then, sections were incubated in secondary antibody (donkey anti-rabbit biotin-conjugated, Jackson ImmunoResearch 711-065-152, RRID:AB_2340593) 1:125 in blocking buffer for 2 h and tertiary antibody solution (streptavidin-horseradish peroxidase conjugated, 1:250 in blocking buffer, Sigma-Aldrich, RPN1231V) for 2 h before being exposed to DAB (Vector Laboratories SK-4100, RRID:AB_2336382) for 10 min. Hematoxylin counterstaining was conducted using the hematoxylin and eosin (H&E) Staining Kit (Abcam, ab245880) as per manufacturer's instructions. Stained sections were dehydrated in a series of ethanol and xylenes solutions, followed by

mounting sections with Permount (Fisher Scientific, SP15-100) and covering with coverslips. <https://doi.org/10.17504/protocols.io.b5s9q6h6>.

H&E staining was performed by the Louise Pelletier Histology Core Facility at the University of Ottawa with a Leica Autostainer XL (Leica Biosystems Inc, Concord, ON, Canada). After deparaffinization, sections were exposed to hematoxylin for 7 min and eosin for 30 s. Then, sections were dehydrated, mounted, and covered with coverslips.

For immunofluorescence antibody staining, tissue sections and primary neurons were incubated in primary antibody (Synapsin, 1:2000, Thermo Fisher Scientific A-6442, RRID:AB_2536207, PSD95, 1:500, Synaptic Systems 124011, RRID:AB_10804286, pSer129 α-Syn, 1:500, Abcam ab51253, RRID:AB_869973, Map2, 1:5000, Abcam ab5392, RRID:AB_2138153, α-Syn, 1:1000, BD Biosciences 610787; RRID:AB_398108, Franklin Lakes, NJ, USA, C-terminally truncated (CTT) α-Syn at residue 119, 1:1000, Roche, asyn-131, CTT α-Syn at residue 122, 1:1000, Roche, asyn-134) in blocking buffer overnight at 4 °C. Next, sections/neurons were incubated in secondary antibody (goat anti-rabbit IgG (H + L) Alexa Fluor™ 647, Thermo Fisher Scientific, A-21244, RRID:AB_2535812, for Synapsin, 119 and 122 CTT α-Syn, goat anti-mouse IgG (H + L) Alexa Fluor™ 488, Thermo Fisher Scientific, A-11001, RRID:AB_2534069, for PSD95 and α-Syn, goat anti-rabbit IgG (H + L) Alexa Fluor™ 488, Thermo Fisher Scientific, A-11008, RRID:AB_143165, for pS129 α-Syn and goat anti-chicken IgY (H + L) Alexa Fluor™ 647, Thermo Fisher Scientific, A-21449, RRID:AB_2535866, for Map2, all at 1:500) together with DAPI (Sigma-Aldrich, D9542) for 1 h at RT, followed by mounting sections with fluorescence mounting medium (Agilent, S302380-2). Brightfield and epifluorescence micrographs were acquired using an Axio Scan Z1 Slide Scanner (Carl Zeiss AG, Oberkochen, Baden-Württemberg, Germany) (20x objective, Louise Pelletier Histology Core Facility) and a Zeiss AxioImager M2 (10x objective, Cell Biology and Image Acquisition Core Facility, University of Ottawa) and analyzed using ImageJ (<https://imagej.net/>, version 1.52p, RRID:SCR_00307) with 2-3 sections per mouse by a blinded investigator. Heatmaps were generated by counting pS129 α-Syn-positive cells in defined regions of the brain (<http://portal.brain-map.org>), normalized to the area occupied by the region and resulting cell densities were expressed as hues of red. Micrographs of in situ hybridization experiments for the expression of *Snca* (experiment 79908848) and *Cdk14* (experiment 71670684) in the mouse brain were downloaded from the Allen Brain Atlas (<http://portal.brain-map.org/>, RRID:SCR_017001) on August 3, 2022.

Cell culture

Cells were kept at 37 °C, 5% O₂, 10% CO₂. HEK293T cells (ATCC, CRL-3216, RRID:CVCL_0063) were cultured in Dulbecco's modified Eagle's medium (DMEM, Wisent Bioproducts, 319-015-CL), supplemented with 10% FBS (Sigma-Aldrich, F1051) and antibiotic/antimycotic (Thermo Scientific, 15240062). For primary mouse cortical neurons coverglass #1.5 (Electron Microscopy Sciences, Hatfield, PA) were coated for at least 16 h at 37 °C, washed twice with ddH₂O and air dried at RT. Mouse embryos at embryonic days 14 to 16 were collected from pregnant females from *Cdk14*^{+/-} crossings (anesthetized with 120 mg/kg Euthanyl). Embryos were decapitated, brains removed, cortices isolated in Hank's Balanced Salt Solution (HBSS) (Millipore Sigma, H9394-500ML) and dissociated in 0.7 mg/ml trypsin (Sigma-Aldrich, T4549). Cell suspensions were quickly washed with 150 µg/ml trypsin inhibitor (Roche, Basel, Switzerland, 10109878001) and 666.67 µg/ml DNase 1 (Sigma-Aldrich, DN25-10mg), followed by another wash with 10 µg/ml trypsin inhibitor and 83.34 µg/ml DNase 1 and resuspended in neuronal media (Neurobasal Media (Thermo Fisher Scientific, Gibco 21103049), 1X B27 supplement (Gibco, 17504044), 1X N2 supplement (Thermo Fisher Scientific, Gibco 17502-048), 1X Penicillin Streptomycin (Thermo Fisher Scientific, Gibco SV30010), and 0.5 mM L-glutamine (Wisent Bio Products, Saint-Jean-Baptiste, QC, Canada, 609-065-EL). Single cells were seeded at ~10⁵ cells per coverslip and maintained in culture for 9 (Figs. S1D and S2B) or 21 days (Fig. 2). Neurons were treated with 2 µg/mL of sonicated human (StressMarq, Victoria, BC, Canada, SPR-322) or mouse α-Syn PFFs (StressMarq, Victoria, BC, Canada, SPR-324) at 2 (Figs. S1D and S2B) or 7 days in vitro (DIV) (Fig. 2). Fourteen days after adding PFFs to the neuronal media, media was extracted and passed through a 40 µm cell strainer (VWR, Radnor, PA, USA, 21008-949) for in vitro α-Syn spreading experiments (Fig. 2). This conditioned media was added to untreated, naive wild type (WT) cortical neuron cultures at the time of the media change (7 DIV) and collected for immunofluorescence experiments at 21 DIV. Neurons treated with conditioned media were analyzed by immunofluorescence quantification after 14 days of treatment at 21 DIV. For immunofluorescence staining, cells were fixed in 4% PFA for

20 min, washed in PBS, and incubated in blocking buffer for 1 h. Cells were incubated with primary antibodies overnight at 4 °C, followed by incubation with secondary antibodies for 1 h at RT.

hiPSC culture, neuronal differentiation, and Cas9-mediated gene editing

Human induced pluripotent stem cell (hiPSC) isogenic lines (Female) and human embryonic stem cell (hESC) isogenic lines (Male) were generated as described previously [34]. hiPSCs were cultured as previously described [35] with slight modifications. Briefly, pluripotent cells were plated in mTeSR (Stem Cell Technologies, Vancouver, BC, Canada) and media was changed daily. The colonies were manually passaged weekly. Differentiation of hPSCs into A9-type DaNs was performed by following a floor plate differentiation paradigm [35, 36]. Immediately preceding differentiation, the colonies were dissociated into a single-cell suspension using HyQTase. hPSCs were collected and re-plated at 4×10^4 cells/cm² on Matrigel (BD Biosciences)-coated tissue culture dishes for differentiation. Floor-plate induction was carried out using hESC-medium containing knockout serum replacement (KSR), LDN193189 (100 nM), SB431542 (10 µM), Sonic Hedgehog (SHH) C25II (100 ng/mL), Purmorphamine (2 µM), Fibroblast growth factor 8 (FGF8; 100 ng/mL), and CHIR99021 (3 µM). On day 5 of differentiation, KSR medium was incrementally shifted to N2 medium (25%, 50%, 75%) every 2 days. On day 11, the medium was changed to Neurobasal/B27/Glutamax supplemented with CHIR. On day 13, CHIR was replaced with Brain Derived Neurotrophic Factor (BDNF; 20 ng/mL), ascorbic acid (0.2 mM), Glial Derived Neurotrophic Factor (GDNF; 20 ng/mL), transforming growth factor beta 3 (TGFβ3; 1 ng/mL), dibutyryl cAMP (dbcAMP; 0.5 mM), and DAPT (10 µM) for 9 days. On day 20, cells were dissociated using HyQTase and re-plated under high cell density 4×10^5 cells/cm² in terminal differentiation medium (NB/B27 + BDNF, ascorbic acid, GDNF, dbcAMP, TGFβ3 and DAPT) also referred to as DA Neuron (DAN)-Medium, on dishes pre-coated with poly-ornithine (15 µg/mL)/laminin (1 µg/mL)/fibronectin (2 µg/mL). Cells were differentiated for up to 60 DIV, with analysis being performed at DIV 14, DIV 45 and/or DIV 60. At D10D and/or D14D of differentiation, hiPSC cultures were transduced with lentivirus containing Cas9 (lentiCRISPR v2, addgene, RRID:Addgene_52961) with the following gRNAs: non-targeting (5'-CGCTTCCGCGCCGTTCAA-3'), CDK14 exon 3 (5'-GCAAAGAGTCACC-TAAAGTT-3') and exon 8 (5'-TGTGCAAAATATAACGCTGG-3'). From D10D-D14D media was supplemented with 0.1 µM compound E (AlfaAesar, J65131, Haverhill, MA, USA). At D18D, cells were re-plated onto poly-ornithine (15 µg/mL)/laminin (1 µg/mL)/fibronectin (2 µg/mL) coated plates. Cells were maintained in DAN-medium (DMEM/F12, 200 µM Ascorbic Acid, 0.5 mM dbcAMP, 20 ng/mL BDNF, 20 ng/mL GDNF, 1 ng/mL TGFβ3, and 1% Anti-Anti) for 6–7 weeks where lysates were then collected for protein analysis.

hESC culture, neuronal differentiation, and in vitro CDK14 inhibitor treatment

Neural progenitor cell (NPC) differentiation was performed as previously described [37]. Dishes for hESC cultures were coated with 0.15 mg/mL growth factor reduced Matrigel (Corning Inc, 354230, Corning, NY, USA) in DMEM/F-12 (Thermo Fisher Scientific, 11320033) for 1 h at RT prior to cell seeding. H9 hESCs (WiCell, WA09, Madison, WI, USA) were seeded as colonies and maintained in mTeSR Plus (StemCell Technologies, 05825). NPC differentiation was initiated when the hESC cultures reached 90% confluence, by replacing growth medium with Knockout Serum Replacement (KSR) medium (414 mL Knockout-DMEM (Thermo Fisher Scientific, 10829018), 75 mL Knockout-serum replacement (Thermo Fisher Scientific, 10828028), 5 mL Glutamax (Thermo Fisher Scientific, 35050061), 5 mL MEM-NEAA (Thermo Fisher Scientific, 11140050), 500 µL 2-mercaptoethanol (Thermo Fisher Scientific, 21985023), 500 µL Gentamicin (Wisent Bioproducts 450-135), 10 µM SB431542 (Tocris, 1614, Bristol, UK) and 500 nM LDN-193189 (Stemgent 04-0074, Reprocell Inc, Yokohama, Kanagawa 222-0033, Japan). Differentiation medium was replaced daily on days 4 and 5 by 75:25 KSR:N2 medium (486.5 mL DMEM/F-12 (Thermo Fisher Scientific, 11320033), 5 mL 15% glucose, 5 mL N2 supplement (Thermo Fisher Scientific, 17502048), 500 µL 20 mg/mL human insulin (Wisent Bioproducts, 511-016-CM), 2.5 mL 1 M HEPES (Thermo Fisher Scientific, 15630080), 500 µL Gentamicin), on days 6 and 7 by 50:50 KSR:N2, on days 8 and 9 by 25:75 KSR:N2 and on days 10 and 11 by N2 medium containing 500 nM LDN-193189. On day 12, differentiated NPCs were treated with Y-27632 (Tocris, 1254) for 4 h, dissociated with Accutase

(Stemcell Technologies, 07922) and seeded into Matrigel coated dishes containing Neural Induction Medium (NIM, 244 mL DMEM/F12, 244 mL Neurobasal medium (Thermo Fisher Scientific, 21103049), 2.5 mL N2 Supplement, 5 mL B-27 Supplement (Thermo Fisher Scientific, 17504044), 2.5 mL GlutaMAX™ (Thermo Fisher Scientific, 35050061), 125 µL 20 mg/mL human insulin, 500 µL 20 µg/mL FGF2 (StemBeads, SB500, Rensselaer, NY, USA), 10 µL 1 mg/mL hEGF (Millipore Sigma E9644) and 500 µL Gentamicin) for expansion. NPCs were passaged at full confluence a minimum of one time before neuronal differentiation.

For NPC-neuronal differentiation culture dishes were coated with 0.001% Poly-L-ornithine (Millipore Sigma, P4957) at 4 °C overnight, followed by 25 µg/mL laminin (Millipore Sigma, L2020) for 2 h at room temperature. NPCs were treated with Y-27632 for 4 h, dissociated with Accutase and seeded at a density of 20,000 cells/cm² in NIM. Neuronal differentiation was initiated when NPCs reached 70% confluence by replacing growth medium with neuronal differentiation medium (244 mL DMEM/F-12 medium, 244 mL Neurobasal medium, 2.5 mL N2 supplement, 5 mL B27 supplement, 200 µL 50 µg/mL BDNF (Peprotech, 450-02), 200 µL 50 µg/mL GDNF (Peprotech 450-10, Thermo Fisher Scientific), 250 mg dibutyryl cyclic-AMP (Millipore Sigma, D0627), 500 µL 100 M L-ascorbic acid (FujiFim Wako Chemicals, 323-44822, Osaka, Japan), and 500 µL Gentamicin). Cells were fed every 3 days for 18 days to obtain immature neuronal networks. FMF-04-159-2 was dissolved in DMSO (Fisher Scientific, BP231) and applied in cell culture medium to hESC-derived neurons for 6 days (with a replenishment of FMF-04-159-2 -containing medium after the first 3 days) and to HEK293T cells for 18 h. HEK293T cells were treated with 500 nM of FMF-04-159-2 together with 200 nM of Bafilomycin A1 (in DMSO, NEB, 546455) or 1 µM of MG-132 (in DMSO, EMD Millipore, 474790) for 18 h to inhibit autophagy or the UPS, respectively. For protein analysis cells were washed with cold PBS, scraped, and collected in low protein binding microcentrifuge tubes (Thermo Scientific, 90410). Cells were pelleted by centrifugation at $1000 \times g$ for 5 min at 4 °C. The supernatant was aspirated, and the cells were lysed in cold RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate; 1% NP-40, 5 mM EDTA, pH 8.0) with protease and phosphatase inhibitors. Cell lysates were incubated on ice for 20 min, with vortexing every 5 min. Lysates were centrifuged at $18,000 \times g$ for 20 min at 4 °C to pellet cell debris. Autophagic activity was monitored by immunoblots for LC3B (Novus Biologicals, NB100-2220). Proteasome activity was monitored by immunoblots for poly-ubiquitinated proteins (anti-ubiquitin P4D1, Cell Signaling Technology, 3136).

Immunoprecipitation

HEK293T cells were transfected for immunoprecipitation (IP) experiments with 2 µg of SNCA-expressing plasmids (clones of the pDEST-GOI-3xFlag backbone including inserts for WT, A30P, G51D, A53T, and S129A forms of SNCA) using enhanced polyethyleneimine transfection reagent in Opti-MEM™ (Thermo Fisher Scientific, 31985070). After 48 h, cells were collected and lysed with IP Lysis Buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP40, 5% Glycerol). 3% of the sample was collected as 'Input'. The remaining sample was incubated with Anti-FLAG® M2 Magnetic beads (Millipore Sigma, M8823) for 1 h at 4 °C. Beads were pelleted and supernatant collected as 'Flow-through', followed by washing of beads with IP lysis buffer. Elution of protein from the beads was performed by 2X Laemmli buffer (Bio-Rad, 1610747, Hercules, CA, USA) with 20% 2-mercaptoethanol (Bio-Rad, 1610710) for 15 min at 75 °C. CDK14 and FLAG-tagged α-Syn were visualized by immunoblots with antibodies for CDK14 (1:500, Santa Cruz Biotechnology, sc50475, RRID:AB_2163226) and FLAG (1:1000, Sigma-Aldrich F3165, RRID:AB_259529). <https://doi.org/10.17504/protocols.io.q26g7poxqgwz/v1>.

ELISA (enzyme-linked immunosorbent assay)

ELISA α-Syn protein quantification was performed as previously described [38, 39]. 384-well MaxiSorp plates (Nunc, Inc) were coated with capturing antibody (α-Syn, BD Biosciences, 610787, RRID:AB_398108) diluted 1:500 in coating buffer (NaHCO₃ with 0.2% Na₂S₂O₅, pH9.6) overnight at 4 °C. Following 3 washes with PBS/0.05% Tween 20 (PBS-T), plates were blocked for 1 h at 37 °C in blocking buffer (1.125% fish skin gelatin; PBS-T). After 3 washes, samples were loaded in duplicates and incubated at RT for 2 h. Biotinylated hSA4 antibody (in-house antibody, RRID:AB_3095299) was generated using 200 µg Sulfo-NHS-LC Biotin (Pierce, Thermo Fisher Scientific), diluted 1:200 in blocking buffer and added to the plate for 1 h at 37 °C.

Following 5 washes, ExtrAvidin phosphatase (Sigma, E2636) diluted in blocking buffer was applied for 30 min at 37 °C. Color development was carried out by using fast-p-nitrophenyl phosphate (Sigma, N1891) and monitored at 405 nm every 2.5 min for up to 60 min. Saturation kinetics were examined for identification of time point(s) where standards and sample dilutions were in the log phase. <https://doi.org/10.17504/protocols.io.261gedywyv47/v1>.

Primary rat neurons, human α -Syn PFFs, and protein analysis

Cortical neurons were harvested from the E18 Sprague Dawley rat embryos (Charles River, RRID:MGI:5651135). The harvested cortical tissue was digested using 17 U/mg Papain followed by mechanical dissociation by gentle trituration through a glass flamed Pasteur pipet. The cells were seeded into plates coated 24 h prior to dissection with Poly-D-Lysine (0.15 mg/mL). The cells were incubated at 37 °C, 7.5% CO₂ until collection. Every 3 to 4 days, a 50% media change was performed (2% B27 supplement, 1% antibiotic/antimycotic, 0.7% BSA Fraction V, 0.1% β -mercaptoethanol in HEPES-buffered DMEM/F12). Where required, cells were exposed to 100 nM FMF-04-159-2 (Bio-Techne, 7158/10, Minneapolis, MN, USA) dissolved in DMSO, at 14 DIV, and again at the subsequent feed (18 DIV). At 14 DIV, cells were exposed to either 1 μ g/mL human α -Syn PFFs, or 1 μ g/mL monomeric α -Syn. Cell lysates were collected at day 5 post PFF or monomeric exposure. Human α -Syn protein was isolated from BL21-CodonPlus (DE3)-RIPL competent cells (RRID:CVCL_M639) transformed with pET21a- α -synuclein and purified by Reversed-phase HPLC. PFFs were then generated as previously described [40]. Purified α -Syn (5 μ g/mL in PBS) was incubated at 37 °C with constant shaking for 7 days, then aliquot and stored at -80 °C. Prior to use, PFFs were thawed and diluted in PBS, then subjected to sonication (20% amplitude, 30 s; 1 s on, 1 s off) and added to neuronal media for exposure to neurons at a concentration of 1 μ g/mL for 24 h. Following the incubation, cell lysates were collected in 150 μ L ice-cold RIPA buffer containing phosphatase and protease inhibitors (1 mM aprotinin, 1 mM sodium orthovanadate, 1 mM sodium fluoride, and 10 mM phenylmethylsulfonyl fluoride). Samples were homogenized using an 18 G needle, left on ice to rest for 15 min, and then centrifuged at 14,000 $\times$ g to remove any cellular debris. For the soluble fraction, cells were lysed in 1% Triton X-100 in TBS buffer (TXS buffer) and cleared by ultracentrifugation at 100,000 $\times$ g for 30 min. Pellets were washed twice with 1% Tx-100 in TBS, then resuspended in 8 M Urea + 8% SDS in TBS buffer (Urea buffer) to generate the insoluble fraction. Using the BioRad DC Protein Assay kit, the protein concentration of each sample was quantified following the manufacturer's guidelines. SDS-PAGE was performed using 12.5% resolving gels and 4% stacking gels, and gels were run for 15 min at 80 V followed by approximately 1.5 h at 110 V. The gels were transferred onto 0.2 μ m nitrocellulose membranes at 35 V and 4 °C overnight. Following the transfer, the membranes were blocked for 1 h at room temperature using blocking buffer (5% non-fat dry milk in 1 X TBST) with constant agitation. Primary antibodies were prepared in blocking buffer containing 0.1% Tween 20 and were probed overnight at 4 °C under constant agitation (CDK14, 1:1000, Santa Cruz Biotechnology sc-50475, RRID:AB_2163226; TH, 1:1000, Pel-Freez Biologicals P40101, RRID:AB_2313713; α -Syn, 1:1000, BD Biosciences, 610787, RRID:AB_398108; pS129 α -Syn, 1:500, Abcam, ab51253, RRID:AB_869973; β -Actin, 1:1000, rabbit, BioLegend, 622101, RRID:AB_315945 or mouse, Sigma-Aldrich A5441, RRID:AB_476744, β -Tubulin, 1:5000, rabbit, BioLegend 802001, RRID:AB_2564645). Following primary antibody incubation, membranes were rinsed using 1XPBS containing 0.1% Tween 20 and subsequently re-blocked using the blocking buffer. The membranes were then probed with secondary antibody for 1 h at RT in blocking buffer containing 0.1% Tween 20 (Goat anti-Mouse IgG (H + L) Secondary Antibody, HRP (Thermo Fisher Scientific 31430, RRID:AB_228307); Goat anti-Rabbit IgG (H + L) Secondary Antibody, HRP (Thermo Fisher Scientific, 31460, RRID:AB_228341); Li-Cor infrared conjugated secondary/IRDye 800RD Donkey anti-Rabbit IgG antibody (LI-COR Biosciences 926-32211, RRID:AB_621843) at dilutions of 1:2000). The membranes were rinsed to remove any residual blocking buffer using 1X PBS containing 0.1% Tween 20. If HRP-conjugated secondary antibodies were used, membranes were probed for 5 min with clarity Western enhanced chemiluminescence blotting substrate (Bio-Rad) and visualized with photosensitive film. For LiCOR-secondary antibodies, membranes were visualized with a LiCOR Odyssey Fc. Total protein was visualized by

Coomassie Brilliant Blue (0.1% Coomassie, 50% Methanol, and 10% glacial acetic acid in ddH₂O).

Statistics

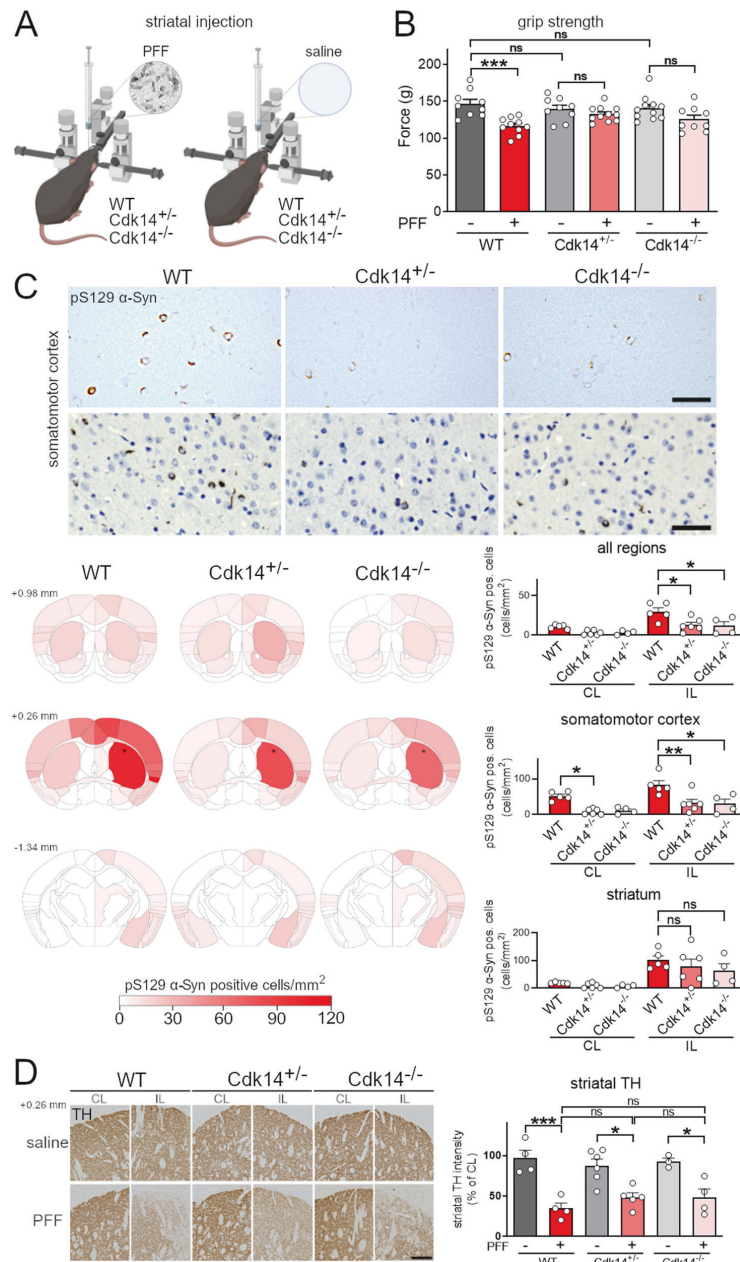
Statistical analysis was performed using GraphPad Prism (<http://www.graphpad.com>, version 9.2.0, RRID:SCR_002798). Quantified data are visualized as mean + standard error of the mean (SEM). Mice were allocated to experimental groups by simple randomization and sample sizes were predetermined based on previous quantification approaches. Investigators were blinded to the group allocation during the execution and the analysis of experiments. Unpaired student's *t* tests were used for two-group comparisons. Data affected by one or two factors were analyzed by one-way or two-way analysis of variance (ANOVA), respectively, followed by Bonferroni *post hoc* comparisons (unless otherwise stated in the figure legend) when at least one of the main factors or the interaction was significant. A significance level of 0.05 was accepted for all tests. Asterisks mark *P* values $\leq$ 0.05 (*), $\leq$ 0.01 (**), $\leq$ 0.001 (***), or $\leq$ 0.001 (****).

RESULTS

CDK14 ablation limits grip strength deficits and reduces cortical α -Syn pathology in PFF-injected mice

We first examined existing *in situ* hybridization data for the expression of *Snca* and *Cdk14* in the murine brain. We observed that both genes are expressed in similar brain regions, including the hippocampus and the SN (Fig. S1A). Since PD is a chronic disease, inhibition of a candidate modifier would have to be safe in the long term. We analyzed Cdk14 protein levels in different mouse organs and tested the effects of Cdk14 depletion on survival, fertility, and organ cytoarchitecture *in vivo*. We found that CDK14 protein is highly abundant in the brain, as well as in the lung and the spleen (Fig. S1B). Cdk14 nullizygous mice are viable, fertile, and exhibited normal brain morphology (Fig. S1C, [28]) and synaptic integrity (Fig. S1D). Furthermore, we did not observe altered morphology of the lung and spleen by Cdk14 ablation (Fig. S1E). We next asked whether silencing *Cdk14* is sufficient to mitigate behavioral and histological phenotypes observed in cultured neurons and in mice exposed to pathogenic α -Syn preformed fibrils (mouse PFFs; Fig. 1A, B; Fig. S2A). 6 months following intrastriatal injection of α -Syn PFFs, there is a stereotypic brain-wide accumulation of pS129 α -Syn—a marker of human synucleinopathies—in addition to SN DaNs loss and mild motor impairments [41–43]. In our experimental paradigm, 6 months after PFF injection (at an age of 12 months), we found that PFF injected WT mice exhibited reduced forelimb force generation in the grip strength test compared to their saline-treated counterparts (Fig. 1B), similar to what has been previously reported [42, 43]. In contrast, the PFF-induced weakening of grip strength was not observed in *Cdk14*^{+/-} or in *Cdk14*^{-/-} mice. We did not observe PFF-mediated changes (in any genotype tested) in the other 8 behavioral tests conducted (including tests for cognitive and motor function). Importantly, we noted that saline-injected *Cdk14*^{+/-} and *Cdk14*^{-/-} mice consistently performed like their WT counterparts in each test, suggesting that chronic Cdk14 reduction is not deleterious to the brain (Fig. S2C).

Next, we analyzed the relative pathological burden of accumulated α -Syn throughout the brain of mice injected with PFFs. We stained for synucleinopathy-linked pS129 α -Syn in the brain of WT mice and found high amounts of pS129 α -Syn-positive cells in the PFF-injected hemisphere (ipsilateral to the injection, IL), which were absent in saline-injected controls (data not shown). We then mapped the distribution of α -Syn pathology at three different rostrocaudal levels near the injection site (relative to bregma: +0.98 mm, +0.26 mm, and -1.34 mm) and found an overall blunting of pS129 α -Syn-positive pathology in the PFF-injected *Cdk14*^{+/-} and *Cdk14*^{-/-} mice compared to their WT littermates (Fig. 1C). This was particularly evident at anatomical regions distal from the



injection site, such as the somatomotor cortex (bregma +0.26 mm) and in other cortical areas. Surprisingly, α-Syn pathology proximal to the injection site in the striatum was not significantly affected between PFF-injected genotypes (Fig.

1C). Similarly to cortical regions of PFF-injected *Cdk14*^{-/-} mice, we observed fewer pS129 α-Syn-positive neurons in *Cdk14*^{-/-} mouse primary cortical cultures treated with PFFs compared to their WT littermate controls (Fig. S2B).

Fig. 1 Loss of *Cdk14* ameliorates grip strength impairment and α -Syn pathology in PFF-injected mice. **A** Mouse α -Syn PFFs were injected unilaterally into the striatum by stereotactic injection in 6-month-old mice. **B** Loss of forelimb grip strength at 6 months post α -Syn PFF-treatment in WT mice, but not in *Cdk14*^{+/-} or *Cdk14*^{-/-} mice. Mean $\pm$ SEM, two-way ANOVA, Bonferroni post hoc, *n*: 9–10. **C** α -Syn PFF-injection increased the load of pS129 α -Syn-positive cells (depicted without and with hematoxylin counterstaining, 50 μ m scale bars) in the injected hemisphere (ipsilateral, IL; injection site indicated by *). Densities of pS129 α -Syn-positive cells are represented in heat maps as hues of red at 3 rostrocaudal levels (relative to bregma: +0.98 mm, +0.26 mm, and -1.34 mm) with dark shades of red correlating to high cell densities (injection sites marked by asterisks). Amounts of pS129 α -Syn-positive cells in all brain regions (averaged in IL and contralateral to the injection, CL), the somatomotor cortex and the striatum at +0.26 mm relative to bregma of PFF-injected *Cdk14*^{+/-} and *Cdk14*^{-/-} mice were lower relative to their wild-type (WT) counterparts. Mean $\pm$ SEM, two-way ANOVA, Bonferroni post hoc comparisons, *n*: 3–6. **D** PFF injection reduces the tyrosine hydroxylase (TH)-positive fiber density IL in comparison to the non-injected hemisphere (CL) to a similar degree in WT, *Cdk14*^{+/-}, and *Cdk14*^{-/-} mice at +0.26 mm relative to bregma (200 μ m scale bar). Mean $\pm$ SEM, two-way ANOVA, Bonferroni post hoc comparisons, *n*: 3–7.

Previous reports have shown that α -Syn PFFs can induce nigrostriatal degeneration over time [41–43]. We stained for tyrosine hydroxylase (TH) at the injection site in the striatum and observed a similar decrease of TH-positive dopamine fibers in WT, *Cdk14*^{+/-}, and *Cdk14*^{-/-} mice injected with PFFs ipsilateral to the injection, relative to their saline-treated counterparts (Fig. 1D). PFF-induced loss of dopamine fibers was not accompanied by altered striatal dopamine content (Fig. S2D). TH staining in the midbrain revealed equal loss of SN DaNs in PFF-injected WT, *Cdk14*^{+/-}, and *Cdk14*^{-/-} mice in comparison to saline-injected controls (Fig. S2D). Together, these data show that loss of *Cdk14* blunts cortical α -Syn histopathology and PFF-induced grip strength impairment without evidently halting nigrostriatal neurodegeneration in the PFF model. It was interesting to note that the effect of *Cdk14* on α -Syn was selective to pathological forms of the protein, as partial reduction or ablation of *Cdk14* in *Cdk14*^{+/-} or *Cdk14*^{-/-} mice, respectively, did not change levels of endogenous mouse α -Syn (Fig. S2E).

Cdk14 loss decreases α -Syn cell-to-cell spread in cortical neuron culture

Our in vivo data suggest that decreasing *Cdk14* reduces distal (e.g., contralateral; higher cortical area) pathology, while not affecting pathology close to the injection site. We hypothesized that *Cdk14* may preferentially affect the cell-to-cell spread of α -Syn, thus contributing to this phenomenon. We assessed the presence of exogenous α -Syn in media of *Snca*^{-/-} mouse primary neurons treated with mouse PFFs and could not detect any residual α -Syn content in the media 14 days post PFF application (without media change, Fig. 2A). Human α -Syn PFFs treated on mouse neurons behaved similarly (Fig. S3A). Thus, α -Syn in the media at 14 DIV originates from cultured neurons (similar to [44]). To analyze if *Cdk14* modifies the cell-to-cell spread of α -Syn, we treated neurons of WT, *Cdk14*^{+/-}, and *Cdk14*^{-/-} embryos with mouse PFFs and collected media 14 days post PFF application (schematic in Fig. 2B). We applied this filtered, seed-competent media to naive WT cultures to test the seeding capacity of α -Syn and found that loss of *Cdk14* dramatically reduced α -Syn pathology (measured by pS129 α -Syn accumulation), 14 days following media application (Fig. 2C).

Knockdown of CDK14 decreases pS129 α -Syn levels in human neurons

Having observed the benefits of *Cdk14* depletion in the PFF mouse model of PD, we next tested whether this benefit translates to human neurons. We infected DaNs derived from a PD patient carrying an A53T mutation in α -Syn [34] as well as its isogenic control with lentiviruses carrying Cas9/sgRNAs against *CDK14*. Neurons infected with sgRNAs targeting either exon 3 (E3) or exon 8 (E8) of *CDK14*, exhibited approximately 50% of the *CDK14* levels of the control cultures (Fig. 3). We found that A53T mutant cells show a marked increase of pS129 α -Syn compared to isogenic controls, and that *CDK14* knockdown significantly lowers pS129 α -Syn levels (Fig. 3). To test whether this effect is

direct we explored the possibility that CDK14 and α -Syn form a complex. We transfected HEK293T cells with FLAG-tagged α -Syn (WT and pathology-associated mutant forms) and performed immunoprecipitation experiments. We did not detect any CDK14 in the precipitated α -Syn protein fraction, suggesting that CDK14's effects on α -Syn are not mediated by a direct physical interaction between both proteins (Fig. S3B). Consistent with this, α -Syn does not appear to have a consensus CDK14 phosphorylation motif.

Pharmacological targeting of CDK14 decreases α -Syn levels and mitigates its pathogenic accumulation

As kinases are typically druggable targets which can be inhibited in non-invasive ways [45], we next asked whether CDK14 inhibition would be a tractable route for decreasing α -Syn levels. We used a recently developed CDK14 covalent inhibitor (FMF-04-159-2) [27] to test whether acute inhibition of CDK14 reduces α -Syn levels. We treated hESC-derived cortical neurons with the CDK14 inhibitor for 6 days and observed a dose-dependent reduction in total α -Syn concentration by ELISA (Fig. 4A). Similarly, we noticed a strong reduction of endogenous α -Syn protein levels by applying the CDK14 inhibitor to HEK293T cells (Fig. S3C), which was accompanied by a reduction of phosphorylated (pS1490) LRP6 (Fig. S3C), a known target of CDK14 [27, 46]. Interestingly, we noted that CDK14 pharmacological inhibition caused its subsequent clearance (Fig. S3C). We asked whether this was mediated by the ubiquitin-proteasome system (UPS) or autophagy, and found that blocking the UPS partially restored CDK14 levels upon its inhibition. On the other hand, we observed that inhibition of autophagy while blocking CDK14 did not affect CDK14 levels (Fig. S3D). We next tested whether the CDK14 inhibitor affects the α -Syn load in rat primary neuronal cultures treated with α -Syn PFFs. Here, PFF treatment induced a spike in insoluble (Urea buffer-soluble) α -Syn protein, which CDK14 inhibition markedly reduced (Fig. 4B). Interestingly, the PFF treatment also induced an increase of CDK14 insoluble protein, which was not present in untreated neurons or in neurons treated with α -Syn monomers (Fig. 4B).

In vivo inhibition of CDK14 decreases the load of human α -Syn

Since the treatment of human neurons and PFF-challenged rat neurons with the CDK14 inhibitor showed a reduction in total and insoluble α -Syn protein, respectively, we next tested whether pharmacological inhibition of CDK14 modifies α -Syn levels in vivo. We administered FMF-04-159-2 via intracerebroventricular infusion at 0.35 mg/kg/day for 28 days in 4-month-old PAC α -Syn^{A53T} TG mice (Fig. 5A; these mice harbor the PD-associated A53T mutant human α -Syn gene in the absence of mouse *Snca* [29]). Administration of the CDK14 inhibitor did not modify body weight development, nor induce any signs of distress or pain as indicated by alterations of locomotion, facial expression, or coat condition of PAC α -Syn^{A53T} TG mice in comparison to vehicle-treated counterparts (Fig. S4A). Similarly, CDK14 inhibitor treatment did not induce changes in the cytoarchitecture of the lung, spleen, and liver (Fig. S4B). To quantify levels of pathogenic forms α -Syn, we collected

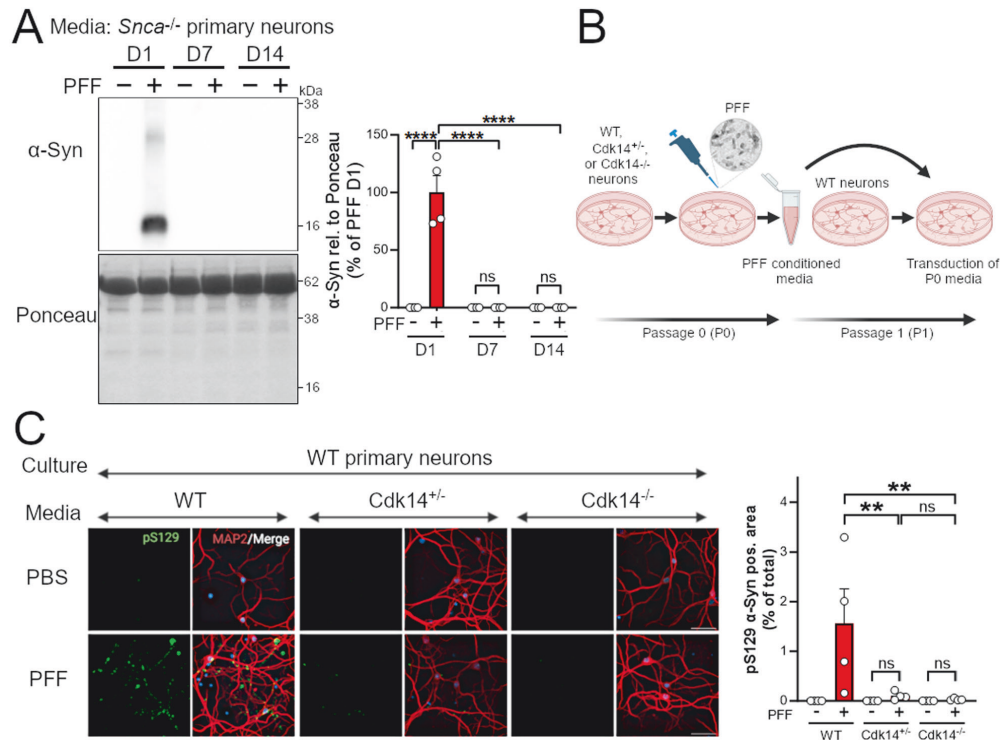


Fig. 2 Primary cortical neurons lacking *Cdk14* exhibit decreased spreading of seed-competent α -Syn. **A** α -Syn is not detectable in media of *Snca*^{-/-} mouse neurons after 14 days (D14) of treatment with mouse α -Syn PFFs. Mean $\pm$ SEM, two-way ANOVA, Bonferroni post hoc comparisons, $n = 4$. **B** Paradigm for passing α -Syn PFF-transduced WT, *Cdk14*^{+/-}, and *Cdk14*^{-/-} primary culture media to WT, naive cultures. Media from 21 DIV cultures transduced with mouse PFFs for 14 days (without media change) were collected, filtered, and added to naive cultures at 7 DIV. Media-treated cultures were then fixed and analyzed for fluorescent pS129 α -Syn signal. **C** Conditioned media from *Cdk14*^{+/-} and *Cdk14*^{-/-} neurons induce less α -Syn pathology (indicated by pS129 α -Syn-positive area) in naive WT neurons compared to WT conditioned media (50 μ m scale bars). Mean $\pm$ SEM, two-way ANOVA, Holm-Sidak post hoc comparisons, $n = 4$.

brains after 1 month of CDK14 inhibitor treatment (Fig. 5A) and analyzed protein content of the TSS, TXS, and SDS buffer-soluble fractions (Fig. 5B). We observed a reduction of total α -Syn and low molecular weight α -Syn species, suggestive of decreased C-terminally truncated (CTT) α -Syn in CDK14 inhibitor-treated mice relative to their vehicle controls. Interestingly, levels of pS129 α -Syn were increased in CDK14 inhibitor-treated mice in the TSS buffer-soluble fraction, whereas it was decreased in the TXS buffer-soluble fraction. Notably, FMF-04-159-2 treatment decreased CDK14 levels (as it did in cells, showing target engagement) in the TXS and SDS buffer-soluble fractions, but not the TSS buffer-soluble fraction. Immunohistology experiments in the hippocampus again displayed a reduction of total α -Syn and a mild, but significant, reduction of two forms of CTT α -Syn (truncations at residues 119 and 122) in CDK14 inhibitor-treated mice (Fig. 5C). Together, these results show that in vivo administration of the CDK14 inhibitor in the brain engages its target and mitigates certain pathogenic forms of human α -Syn in the mouse brain without inducing obvious discomfort or pain.

DISCUSSION

α -Syn is increasingly considered a valid experimental therapeutic target for PD, based on clinical genetic and neuropathological

evidence, as well as animal and cell culture studies. *SNCA* gene mutations or amplifications resulting in α -Syn pathology are tightly linked to PD pathogenesis. Moreover, α -Syn is a major constituent of Lewy-like structures, the pathological hallmark of PD and related synucleinopathies. Thus, targeting α -Syn has been a major thrust in the pharmaceutical realm. One aspect of α -Syn pathology that has been difficult to overcome is the notion that different states of its post-translational modification or aggregation differentially affect disease pathogenesis: a clear image has yet to emerge as to the real culprit of α -Syn toxicity. Although novel strategies such as anti-sense oligonucleotides, immunotherapy and small molecule inhibitors of α -Syn aggregation are being explored [47], finding a target that can be pharmacologically inhibited still holds potential as a minimally invasive and simple strategy to lower α -Syn levels—especially when such treatment course would be made over several decades. Indeed, a growing body of evidence suggests that α -Syn may play a role not only at the presynaptic space but also in the immune system [38, 48, 49]. Therefore, careful titration of its levels may be clinically crucial. As a result, we asked whether candidates that are more amenable to traditional pharmacology (i.e., kinases) could regulate α -Syn dosage, irrespective of its aggregation status. Our previous studies identified a handful of these modifiers including TRIM28 and DCLK1 [8, 22, 50, 51]. Here, we study a heretofore unexplored

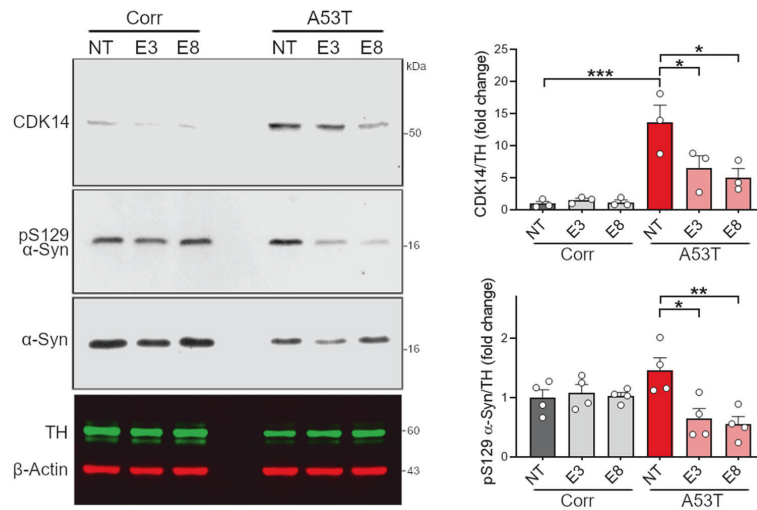


Fig. 3 Genetic reduction of CDK14 attenuates pS129 α-Syn in human neurons. Immunoblots illustrating elevated amounts of pS129 α-Syn in the RIPA buffer-soluble protein fraction of hiPSC-derived *SNCA* A53T human neurons (in comparison to isogenic corrected neurons [Corr]) which is reduced by CRISPR/Cas9-mediated knockdown of *CDK14* (targeted against *CDK14* exon 3 (E3) and 8 (E8), NT non-targeting control). Mean + SEM, one-way ANOVA, Tukey post hoc comparisons, $n = 3-4$.

target as well as its newly developed cognate inhibitor [27] for disease modification in pre-clinical models of PD: CDK14.

We show that the reduction of CDK14 protein levels is well tolerated and causes a reduction in pathogenic α-Syn accumulation in murine and human models of synucleinopathy. Genetic suppression of *Cdk14* reduces pS129 α-Syn pathology in the cortex of PFF-injected mice and dampens the development of grip strength deficits in these mice; while this rescue is not found at sites proximal to the injection, including the nigrostriatal tract. Importantly, the genetic reduction of CDK14 in DaNs derived from an individual with synucleinopathy shows equal promise in preventing phenotypic development. We show that the selective covalent CDK14 inhibitor, FMF-04-159-2, decreases α-Syn levels in hESC-derived human neurons and mitigates PFF-induced α-Syn pathology in rat cortical neurons. Lastly, we demonstrate that administering FMF-04-159-2 in vivo reduces α-Syn dosage and, consequently, decreases pathogenic forms of α-Syn in a humanized mouse line expressing PD-linked A53T *SNCA*. Collectively, these results show that CDK14 is a pharmacologically tractable target for synucleinopathy.

We observed that loss of *Cdk14* in PFF-treated mice reduced the level of α-Syn histopathology in cortical areas, such as the somatomotor cortex. Surprisingly, we did not detect changes in the load of pS129 α-Syn-positive cells by *Cdk14* ablation in the striatum, the PFF injection site (Fig. 1C). In line with this, we noticed a similar PFF-induced degeneration of the dopaminergic nigrostriatal system in WT, *Cdk14*^{+/−}, and *Cdk14*^{−/−} mice (Fig. 1D and Fig. S2D). These observations imply that loss of CDK14 reduces the degree of intercellular α-Syn spreading rather than protecting neurons which are directly exposed to PFFs. Indeed, when we tested this in a cultured neuron system, we found that genetic reduction of *Cdk14* dramatically decreased the spreading capacity of seed-competent α-Syn (Fig. 2). Therefore, it is plausible that CDK14 facilitates the cell-to-cell spread of α-Syn. Exosomal α-Syn is more likely to be taken up by recipient neurons than free extracellular α-Syn [52]. However, the precise mechanism of α-Syn loading into exosomes and their release is not fully understood [52, 53]. Further investigations will address if CDK14 affects exosomal α-Syn release. Genetic reduction of CDK14 using a

CRISPR/Cas9-mediated strategy in stem cell-derived human neurons carrying the PD-linked *SNCA* A53T mutation lowered levels of pS129 α-Syn (Fig. 3), indicating that ablation of both, murine and human *Cdk14/CDK14* mitigates the neuronal load of this pathology-linked form of α-Syn.

Given the lack of phenotypes in the *Cdk14*^{−/−} or *Cdk14*^{+/−} mice, no evidence for loss of function intolerance in humans (probability of loss of function intolerance [pLI] = 0; gnomAD database, *CDK14* | gnomAD v2.1.1) [54], and the availability of a recently developed highly selective CDK14 inhibitor, we further explored the pharmacological tractability of CDK14 in the context of synucleinopathy. Treatment of hESC-derived cortical neurons with the newly developed CDK14 inhibitor FMF-04-159-2 induced a pronounced reduction of the total α-Syn concentration, as measured by ELISA quantification (Fig. 4A). FMF-04-159-2 was recently designed to provide an improved pharmacological tool for the inhibition of CDK14 as treatment for colorectal cancer [27]. Similarly to what we observed, this study demonstrated that FMF-04-159-2 reduced CDK14 activity and lowered CDK14 levels [27], potentially by inhibiting its autophosphorylation and promoting its destabilization. Applying the UPS inhibitor MG-132 together with the CDK14 inhibitor to HEK293T cells partially restored CDK14 levels, suggesting that clearance of CDK14 upon its inhibition is promoted by the UPS (Fig. S3D). Interestingly, FMF-04-159-2 was described to covalently bind and inhibit CDK14 at ~100 nM ($IC_{50} = 86$ nM [27]), a dosage which lowered α-Syn levels to ~12% of vehicle-treated controls in our in vitro experiments. Adding the CDK14 inhibitor to PFF-challenged rat cortical neurons reduced the amount of aggregated (Urea buffer-soluble) α-Syn species (Fig. 4B), phenocopying the low degree of pS129 pathology in cortical neurons of PFF-treated *Cdk14*^{−/−} mice (Fig. 1C) or cultures (Fig. 2 and Fig. S2B). Interestingly, we found that *Cdk14* accumulated in the urea-soluble fraction, specifically upon PFF treatment. To our knowledge, it is not known whether CDK14 is present in LBs or LNs in the brains of PD patients. Future histopathology experiments may generate deeper insights into whether CDK14 accumulates, and forms aggregates together with α-Syn in patients with synucleinopathies. Comparable to CDK14

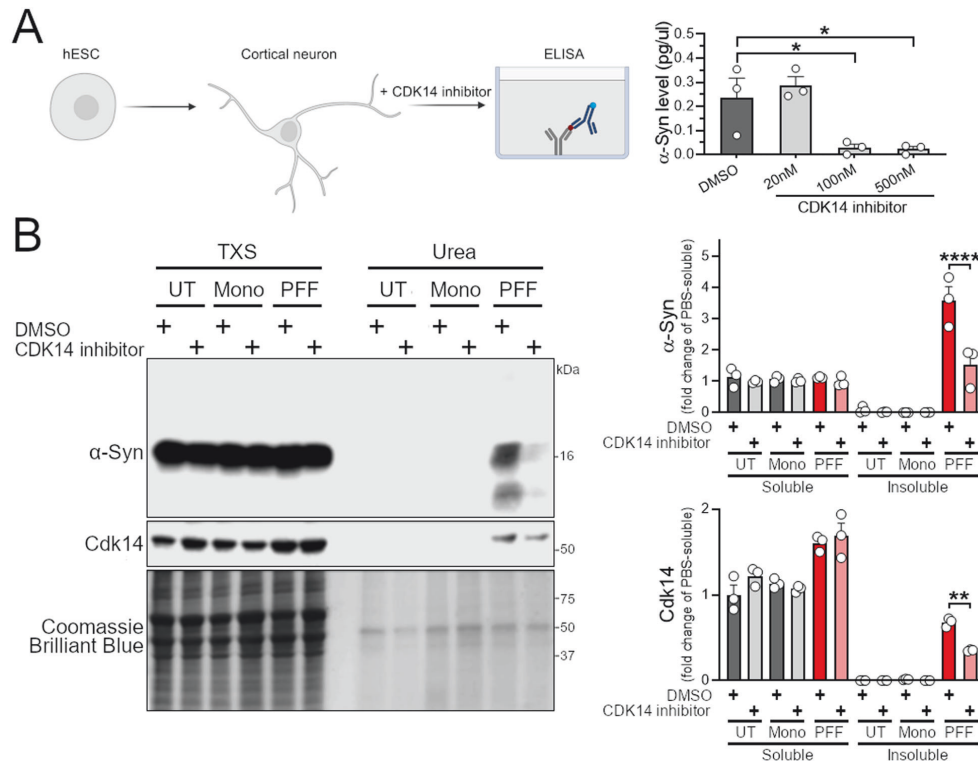


Fig. 4 Pharmacological inhibition of CDK14 reduces α -Syn protein burden in human and rodent neurons. **A** Dose-dependent reduction of α -Syn in hESC-derived human neurons is detected by ELISA-quantification after 6 days of CDK14 inhibitor FMF-04-159-2 treatment. Mean $\pm$ SEM, one-way ANOVA, Bonferroni post hoc comparisons, $n = 3$. **B** Human α -Syn PFFs applied to rat cortical neurons for 5 days increase the amounts of α -Syn and CDK14 in the insoluble protein fraction (Urea buffer-soluble) compared to untreated (UT) and α -Syn monomers (Mono)-treated neurons, as shown by immunoblots, which were reduced by the application of 100 nM of the CDK14 inhibitor. Mean $\pm$ SEM, two-way ANOVA, Holm-Sidak post hoc comparisons, $n = 3$.

inhibitor-treated rat neurons with PFFs, we observed a reduction of total α -Syn in the more insoluble, TXS buffer-soluble protein fraction (and in the TSS buffer-soluble fraction) of PAC α -Syn^{A53T} TG mice which received FMF-04-159-2 via intracerebral injection (Fig. 5A, B). Reduction of total α -Syn was accompanied by lower amounts of CTT α -Syn (Fig. 5B, C), indicating that this form of α -Syn, which increases α -Syn's propensity to aggregate and enhances its cytotoxic effects [55–57], is modulated by CDK14. Levels of pS129 α -Syn were reduced in the more insoluble TXS fraction of CDK14 inhibitor-treated mice, again pointing towards lower degrees of aggregated, pathology-relevant forms of α -Syn. In contrast to these findings, we found paradoxically increased amounts of pS129 α -Syn in the TSS buffer-soluble fraction (highly soluble) of CDK14 inhibitor-treated mice, suggesting that Cdk14 blockage increased pS129 α -Syn in the cytosol of this α -Syn-humanized mouse line. Notably, we did not observe any macroscopic signs of PD/neuropathy-linked behavioral abnormalities in PAC α -Syn^{A53T} TG mice upon CDK14 inhibitor treatment, such as loss of motor activity or imbalance during locomotion (Fig. S4A), implying that higher amounts of cytosolic pS129 α -Syn do not substantially promote cerebral and functional impairments. Elevated levels of cytosolic, non-aggregated pS129 α -Syn seem to be indicative of earlier stages of synucleinopathies, as cytoplasmic networks positive for pS129 α -Syn are more

commonly observed in neurons without LBs from patients with early-stage disease, than in LB-containing neurons of patients with advanced PD [58]. Based on these *in vivo* CDK14 inhibitor administration experiments, we hypothesize that blockage of CDK14 activity reduces loads of pathology-linked forms of insoluble α -Syn, potentially shifting synucleinopathy progression to an earlier phase of disease development.

Our experiments provide insights into the modulatory effects on α -Syn pathology by reducing CDK14 activity in neurons from multiple mammalian species. However, in this study (Fig. S3B) and previously [22], we did not observe any direct interaction between CDK14 and α -Syn. Moreover, we could not detect any kinase activity of recombinant CDK14 toward wild-type α -Syn *in vitro* (data not shown). We therefore hypothesize that loss or inhibition of CDK14 causes a decrease in α -Syn through unknown mediators, ultimately regulating α -Syn protein levels and intercellular spreading of α -Syn pathology. Low CDK14 activity attenuates Wnt/ β -catenin signaling [46] which is associated with increased activity of glycogen synthase kinase-3 β (GSK-3 β) [59]. Interestingly, high GSK-3 β activity promotes α -Syn accumulation [60]. Future investigations will show if CDK14 inhibition reduces the α -Syn burden by modifying Wnt/ β -catenin signaling and downstream GSK-3 β activity.

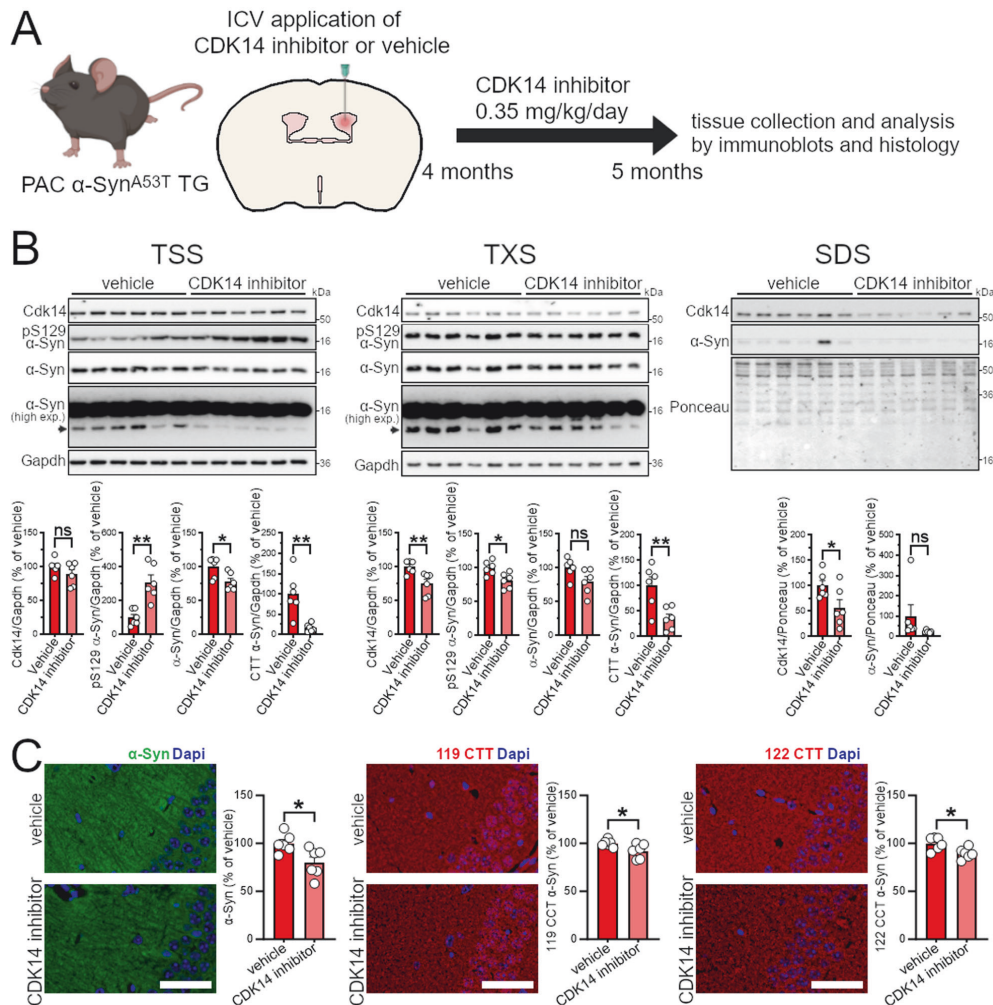


Fig. 5 Inhibition of Cdk14 decreases human α -Syn protein in mice expressing A53T SNCA. **A** Intracerebroventricular (ICV) administration strategy for the CDK14 inhibitor FMF-04-159-2 at 0.35 mg/kg/day for 28 days in 4-month-old PAC α -Syn^{A53T} TG mice. **B** Immunoblots with protein isolated from the brain of FMF-04-159-2-treated PAC α -Syn^{A53T} TG mice visualize the reduction of α -Syn in the TSS buffer-soluble, Cdk14 in the TXS buffer and SDS buffer-soluble and C-terminally truncated (CTT) α -Syn (arrowhead at high exposure (high exp.)) in the TSS buffer- and TXS buffer-soluble protein fraction in comparison to vehicle-treated mice. Mean $\pm$ SEM, unpaired student's *t* test, *n* = 6. **C** Micrographs of the hippocampal CA3 region depict the reduction of α -Syn, 119 and 122 CTT α -Syn in the brain of FMF-04-159-2-treated PAC α -Syn^{A53T} TG mice (50 μ m scale bars). Mean $\pm$ SEM, unpaired student's *t* test, *n* = 6.

Our results examining the genetic and pharmacological reduction of CDK14 in PD models set the stage for future pre-clinical studies. In all behavioral experiments conducted, *Cdk14*^{-/-} mice were indistinguishable from WT mice (Fig. 1B and Fig. S2C). Furthermore, loss of Cdk14 did not alter the architecture of brain tissue or peripheral organs (Fig. S1C–S1E) implying that *Cdk14* loss is not deleterious in vivo. This is supported by human genetics where the loss of CDK14 appears to be well tolerated. Additionally, Cdk14 inhibition in vivo did not induce any signs of discomfort (Fig. S4A), implying that pharmacological targeting of CDK14 is safe. Similarly, systemic administration of FMF-04-159-2 at a ~140-fold higher delivery rate

(50 mg/kg/day) appears to be well tolerated by mice as shown in a recent study [46] where Cdk14 inhibition mitigated the growth of lung tumors. In our study, the activity of the CDK14 inhibitor in human neurons appears to be high, affecting CDK14 (and thus α -Syn metabolism) in the nanomolar range (Fig. 4A). Further in vivo experiments will test whether CDK14 inhibition rescues PD-like neuron loss and behavioral phenotypes in models of synucleinopathies; elucidating the potential of targeting CDK14 as a therapeutic strategy for PD patients.

In sum, we show that CDK14 inhibition causes a decrease of total α -Syn concentrations under both ex vivo and in vivo

conditions, ameliorates the levels of pathology-relevant forms of α -Syn and reduces cell-to-cell transmission of α -Syn. Given the strong evidence linking α -Syn levels to PD pathogenesis, we conclude that targeting CDK14 function holds promise as a potentially disease-modifying approach to treat PD.

CONCLUSIONS

Elevated α -Syn levels are closely linked to PD. In this study, we explore the effect of inhibiting CDK14 as a pharmacologically tractable approach to decrease α -Syn levels in cultured neurons and animal models. We show that the genetic reduction of Cdk14 mitigates grip strength impairment and ameliorates cortical α -Syn pathology in PFF-treated mice, without affecting nigrostriatal pathology proximal to the injection site; Cdk14 likely acts to regulate the intercellular spread of seed-competent α -Syn. Similarly, CDK14 ablation reduces α -Syn pathology in human dopaminergic neurons derived from PD patients. Finally, pharmacological targeting of CDK14 lowers pathological α -Syn in cultured neurons and modifies pathogenic forms of α -Syn in mice expressing PD-linked human A53T SNCA. Taken together, we propose CDK14 inhibition as a novel pre-clinical strategy to modify the progression of synucleinopathies.

DATA AVAILABILITY

All data of this study are in the main text or in the Supplementary Materials. All quantified data have been made public (<https://zenodo.org/records/10654887>, <https://doi.org/10.5281/zenodo.10654887>). Cdk14^{-/-} mice were obtained from David S. Park from the University of Calgary and are on an F14 C57BL/6N background. Snca^{-/-} mice were provided by Matthew Farrer from the University of Florida, Gainesville. PAC α -Syn^{A53T} TG mice (dbl-PAC-Tg(SNCA^{A53T})+/-;Snca^{-/-} [29]) were provided by Robert L. Nussbaum from the University of California, San Francisco. Any other materials are commercially available.

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AUTHOR CONTRIBUTIONS

Conceptualization: JLAP, KMR, and MWCR. Methodology: JLAP, KMR, MS, BBD, EL, BN, NAL, HMG, AB, MGS, BBD, SMC, and MWCR. Investigation: JLAP, KMR, MS, BBD, HMG, SMC, and MWCR. Supervision: WLS, MGS, SDR, and MWCR. Writing—original draft: JLAP, KMR, and MWCR. Writing—review & editing: JLAP, KMR, JTT, MGS, WLS, PB, SDR, and MWCR. Resources: AJ, JM, and PB.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Animal experiments were done under the approved breeding and behavior protocols approved by the University of Ottawa Animal Care Committee. Studies with hESCs were performed following approval by the Stem Cell Oversight Committee of Canada and the Institutional Review Board (Ottawa Health Science Network Research Ethics Board).

ADDITIONAL INFORMATION

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